EFFECT OF BENZOATED BRINE DIPS ON KEEPING QUALITY OF FISH FILLETS[1]

C. R. FELLERS AND E. W. HARVEY
Massachusetts State College, Amherst, Massachusetts

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In spite of the many modern improvements in the technology and distribution of fish products, there are still large areas of the United States where it is difficult to obtain fresh fish of good quality. This fact is particularly true during the warm months of the year. Modern filleting and quick-freezing methods have done a great deal for both the fishing industry and the fish consumer, but expanded markets are still needed. The present investigation was undertaken to ascertain the effect of very small quantities of benzoic acid and certain benzoates when used in sodium chloride brines on the keeping quality and palatability of fish fillets.

PRESENT STATUS OF FISH TREATMENT WITH BENZOATES

Very little work on the use of benzoates in marine products has been reported in the literature. In both the Canadian maritime provinces and New England mixtures of borax and sodium benzoate have been used for many years in the salting of cod and other bottom fish. Some reports have also come from Germany relative to the use of various benzoic acid compounds in the treatment of herrings and other marine products. Bérubé (1938) briefly discussed the preservation of fish in eastern Canada by means of chemicals including benzoic acid. To a limited extent, benzoated-brine dips as well as ice containing .1 per cent sodium benzoate have been used experimentally in treating cod, haddock, and halibut on both the Atlantic and Pacific Coasts. Tarr and Sunderland (1938) of the Canadian Pacific Fisheries Experimental Station at Prince Rupert have reported that a 15-minute dip of the fillets in a 20-per cent salt brine containing .1 per cent of benzoic acid, exerted a favorable influence on keeping quality of lightly smoked fish. The treatment was particularly effective in preventing the formation of the decomposition product, trimethylamine. The action of the benzoic acid was apparently not improved by the addition of citric and lactic acids or of acid potassium phosphate. There was no evidence that benzoic acid and trimethylamine combine to form a stable compound undissoci-

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ated by alkali. Consistent decreases in numbers of bacteria as a result of the benzoate dip were not obtained, but there was a marked effect on keeping quality of the smoked fish in storage. The species used in these Canadian experiments were red snapper, grey cod, ling cod, halibut, and spring salmon. It is of interest to record that 10 parts of trimethylamine nitrogen per 100,000 parts of fish flesh indicates an unpalatable fish, and that at between 20 and 30 parts per 100,000 the fish are considered definitely unfit for human consumption. No maximum counts for bacteria in edible fish have been suggested as sanitary standards. Unpublished data furnished the authors in March, 1939, by R. H. Bedford,[2] formerly of the Prince Rupert

TABLE 1

Effect of Brine, Acid, and Benzoate Dips on Bacterial Counts and Keeping Quality of Haddock Fillets[1]

Treatment	Length of dip	Storage temperature	Bacteria per gram in 48 hours	Time when stale	Time when tainted
	min.	°F.		*hr.*	*hr.*
No brine	10	48	1,128,000	48	66
Salt, 5%	10	48	338,000	66	84
Salt, 5% and phosphoric acid, 1%	10	48	80,500	60	72
Salt, 5% and sodium benzoate, 5%	10	48	69,500	84	96
Salt, 5%, acid 1%, and sodium benzoate, .5%	10	48	45,500	84	108[2]

[1]Average count of fillets before dipping was 13,500 bacteria per gram. [2]Approximate time.

Fisheries Experimental Station, show that in the laboratory and in commercial practice the use of ice containing .1 per cent of benzoic acid has a pronounced antiseptic effect and retards the rate of bacterial growth on halibut. He points out the obvious advantages of early treatment while the fish are still fresh, rather than attempting to treat them after they are already of questionable quality.

EXPERIMENTS WITH BENZOATES

Iced haddock fillets from the Boston Fish Pier were received in good condition in Amherst, eight hours after filleting. They were treated in duplicate as indicated (Table 1). After being drained, the fillets were wrapped in clean parchment and stored away. Bacteriological examinations were made in duplicate of fillets after 48 hours.

This preliminary experiment shows that dipping the fillets in any of the brines significantly reduced the bacterial counts and improved keeping quality. The benzoate seemed to show an effect over

[2] Present address—Atlantic Coast Fisheries Co., New York, N. Y.

and above that of the brine alone or the brine plus phosphoric acid. Some of the benzoated fillets were fried and eaten after 60 hours in storage. The flavor was good with no detectable benzoate taste.

RELATIVE VALUES OF DIFFERENT BENZOATES IN TREATING FILLETS

Pure sodium, magnesium, calcium, and ammonium benzoates, as well as benzoic acid and a mixture of 66.6 per cent benzoic acid and 33.3 per cent sodium benzoate, were compared in effectiveness on the basis of their actual weights in the brine as well as their content of benzoic acid radical. The tests were made on haddock fillets. For bacteriological examination, portions of the fillets were passed through sterile food choppers, as very consistent counts can be secured in this way. Both two-minute and 10-minute dips were used. After being dipped the fillets were momentarily drained, immediately wrapped in parchment paper, and stored at 17.2 to 22.2°C. (63 to 72°F.) for two to five days. To save space the data are omitted because they show no particular differences in values except that the calcium and ammonium benzoate and the benzoic acid (owing to its low solubility) were less effective than the sodium, magnesium, or salt-acid mixture. Growth of bacteria in fish fillets are not entirely inhibited by treatment with these salts. In fact, when the initial contamination was heavy, benzoate-brine dips were far less effective than when good fresh fillets were used. Benzoate concentrations of .05 and .1 per cent were used but were not particularly effective in improving keeping quality. A concentration of .2 per cent exerted definite increased protection against spoilage, and .4 per cent was even better. The two-minute dip was almost as satisfactory as the 10-minute dip. Care must be exercised not to use brines of pH value less than 4, otherwise protein precipitation and color changes will occur.

EFFECT OF BENZOATE DIPS ON BACTERIA AND KEEPING QUALITY OF HADDOCK FILLETS

Since sodium benzoate was more effective as a fish preservative than any of the other benzoate salts tried, in the present series of tests only this salt and a mixture of equal proportions of sodium benzoate and benzoic acid were used. The dipping time was varied from 30 seconds to two minutes and the benzoate concentration (by weight) was varied from .1 to .5 per cent. In this series of dips (Tables 2 and 3) the benzoate was used in a five-per cent brine, while in another series (not given) no salt brine was used. Data on the one-minute dip are omitted but are similar to those in Tables 2 and 3.

TABLE 2

Effect of a Two-Minute Dip in Benzoated Five-Per Cent Salt Brine on Bacterial Count and Keeping Quality of Haddock Fillets

Treatment	pH	Average bacterial count before dip	Average bacterial count after dip	Average bacterial count after storage for 8 days at 3.3°C. (38°F.)	Average keeping quality [1]
		per gm.	*per gm.*	*per gm.*	*days*
Control, not dipped	....	11,000	12,000	29,000,000	4
Sodium chloride, 5%	6.7	10,000	6,000	50,000,000	4
Sodium benzoate, .1%	6.8	12,000	7,200	20,200,000	5-6
Sodium benzoate, .3%	6.8	5,000	3,500	6,200,000	11
Sodium benzoate, .5%	6.8	9,000	3,200	5,100,000	13
Sodium benzoate, .25% and benzoic acid, .25%	3.8	10,000	5,700	5,500,000	22
Sodium benzoate, .15% and and benzoic acid, .15%	3.8	7,500	4,500	24,000,000	14
Sodium benzoate, .05% and benzoic acid, .05%	3.9	8,000	3,600	55,000,000	5

[1] Keeping quality was judged by raw odor and in some cases by flavor after cooking.

TABLE 3

Effect of a 30-Second Dip in Benzoated Five-Per Cent Salt Brine on Bacterial Count and Keeping Quality of Haddock Fillets

Treatment	pH	Average bacterial count before dip	Average bacterial count after dip	Average bacterial count after storage for 6 days at 3.3°C. (38°F.)	Average keeping quality [1]
		per gm.	*per gm.*	*per gm.*	*days*
Control, not dipped	....	22,000	22,000	9,000,000	4-5
Sodium chloride, 5%	6.4	26,000	39,000	3,100,000	5-6
Sodium benzoate, .1%	6.5	36,000	45,000	3,640,000	6-7
Sodium benzoate, .3%	6.6	50,000	66,000	1,650,000	10
Sodium benzoate, .5%	6.6	9,000	37,000	440,000	15
Sodium benzoate, .25% and benzoic acid, .25%	3.8	48,000	18,000	215,000	16
Sodium benzoate, .15% and benzoic acid, .15%	3.8	22,000	17,000	315,000	9
Sodium benzoate, .05% and benzoic acid, .05%	3.8	45,000	43,000	1,010,000	5-6

[1] Keeping quality was judged by raw odor and in some cases by flavor after cooking. In no case was the benzoate taste detected in these cooked fillets.

The data as expressed in the tables are reasonably consistent. There is not, however, a perfect correlation between numbers of bacteria, for example, and keeping quality of the haddock fillets. Similarly, the same concentration of benzoates does not always give the same protective action. However, the results show rather conclusively that haddock fillets dipped for one or two minutes in a five-per cent salt brine containing at least .15 per cent of sodium benzoate or sodium benzoate-benzoic acid mixture will not only materially retard the growth of bacteria but will greatly extend the storage life of the fillet.

In this series of tests two fillets were tested in every case and the average value is given in the tables. The bacteriological counts were made on the standard agar of the American Public Health Association and counted after 48 hours' incubation at 37°C.(98.6°F.). In some cases, the presence of a great many pin-point colonies prevented accurate determinations. It seems likely that benzoates exert a selective action on certain types of bacteria as has been suggested by Tarr and Sunderland in their work on halibut fillets. In general, the reduction in pH value to below 4.5 materially assisted in retarding bacterial growth in the haddock flesh. The appearance of the dipped fillets in the solutions remained unchanged. After being dipped, they were drained of free-run brine, wrapped in waxed paper, and stored in the refrigerator at approximately 3.3°C.(38°F.). The differences in effectiveness between the one-half-, one-, and two-minute dips were not marked. This fact is again indicative that the main action of the salt and benzoate is on the surface of the flesh. The benzoates are distinctly more effective when used in a five-per cent salt brine than when used alone in water.

While definite statements cannot be made, the data indicate that benzoate-salt brine dips can be expected to prolong the storage life of haddock fillets held in cold storage for from three to 10 days. This period should be a material advantage in the shipment of fresh, iced, or refrigerated fillets.

In an effort to determine the bacterial quality of the waxed wrappers used for fillets, 10 samples were carefully examined. The results showed an average of eight bacteria per 60 square centimeters of area. This does not indicate serious contamination.

It is significant that even in the fillets in brine containing .5 per cent sodium benzoate, no judge detected any benzoate taste in the cooked (boiled or fried) fillets.

It was consistently observed that the protective action of the benzoate was greater if odor and taste were used as criteria rather than total numbers of bacteria. This same observation has been

made by Tarr and Sunderland (1938) in Canada. They found that benzoic acid suppressed trimethylamine formation without apparently greatly affecting the bacterial population of halibut and cod fillets. They conclude that the benzoic acid either suppresses only certain species of bacteria or that it merely regulates the metabolism of the organisms.

BENZOATES IN PRACTICAL FISH PACKING

At the Boston Fish Pier cod and red perch fillets were dipped for two-minute periods in the following solutions:

1. .3 per cent sodium benzoate in five-per cent brine and
2. .15 per cent sodium benzoate and .15 per cent benzoic acid in five-per cent brine.

TABLE 4

Bacteria per Gram on Fillets Dipped for Two Minutes and Stored for Five Days at 3.3°C.(38°F.) as Compared With Controls

Treatment	Fillet 1	Fillet 2	Fillet 3	Fillet 4
COD FILLETS				
Control	3,200,000	2,800,000	2,600,000	2,700,000
Sodium benzoate,[1] .3%	520,000	590,000	2,300,000	2,300,000
Sodium benzoate, .15% and benzoic acid, .15%	280,000	500,000	1,400,00	500,000
RED PERCH FILLETS				
Control	1,700,000	1,860,000	2,000,000	2,300,000
Sodium benzoate,[1] .3%	1,720,000	1,980,000	2,300,000	2,400,000
Sodium benzoate, .15% and benzoic acid, .15%	500,000	300,000	1,300,000	1,200,000

[1]All solutions made in five-per cent brine; incubation for 48 hours at 32°C.(89.6°F.).

These pieces were stored for five days in the cold room at 38°F., at which time bacterial counts and organoleptic tests were made; the results of these tests are shown (Table 4).

The cod and perch fillets immersed in the five-per cent brine solution containing .15 per cent sodium benzoate and .15 per cent benzoic acid (total benzoate .3 per cent) were more effectively preserved bacteriologically and organoleptically than either the controls or those dipped in five-per cent brine containing .3 per cent sodium benzoate.

At the same time in Boston, pieces of cod fillets were dipped in these solutions, wrapped in cellophane, packed in boxes, and frozen. These pieces were stored in this condition for four weeks and then removed. Bacterial counts before thawing, after thawing, and after

storage for one and for five days, as well as organoleptic tests after frying, were made (Tables 5 and 6).

Marked bacteriostatic effect is seen in the frozen fillet tests. The bacterial counts show that bacteriostasis takes place (1) before and during freezing, (2) after thawing, and (3) after thawing and storage. Organoleptic observations are correlated with the above results. Before thawing, both the .3 per cent sodium benzoate and the .3 per cent benzoic acid plus sodium benzoate mixture markedly reduced the numbers of bacteria present. In some fillets after treatment the counts were only 10 per cent as high as in the controls. The effect of the benzoate continues after the frozen fillets have been thawed and held in storage. Thus, spoilage is greatly delayed and the period of storage for fillets treated with benzoates can be extended over that for untreated fillets.

COOKING TESTS ON FILLETS DIPPED IN BENZOATED BRINE

Cod fillets were dipped in a five-per cent salt brine containing .3 per cent sodium benzoate, wrapped separately after five days' storage at 38°F., and cooked (boiled and fried) for flavor tests. Comparisons were made with similar fillets dipped in a benzoated brine containing .15 per cent each of sodium benzoate and benzoic acid, as well as with untreated controls (Table 5).

With reference to Table 4, again the beneficial action of benzoates in extending the keeping of cod fillets is observed. By dipping the fillets to be frozen in a benzoated brine, the storage life and bacteriological count of the defrosted fillets were materially improved. The benzoic acid-sodium benzoate mixture (total .3 per cent) was most effective. The benzoated fillets kept perfectly for five days after defrosting and were still edible after about nine to 10 days at 38°F.

All odor and taste tests, upon cooking the fillets after five days' storage, showed no spoilage after five days, and no difference was noted by the taste panel in flavor between the treated and untreated fillets. A suggestion of benzoate taste was noticed by two observers in the fillets treated with equal parts of sodium benzoate and benzoic acid.

After 12 days in storage at 38°F., the tests were repeated. The controls were definitely inferior to the treated fillets both in odor and flavor; in fact they had begun to spoil. On the other hand, the benzoated fillets seemed to be in good condition and were eaten without adverse comment. The mixture of benzoic acid and sodium

TABLE 5

Bacteria per Gram on Frozen Cod Fillets Before Thawing and After Four Weeks' Storage[1]

Treatment	Fillet 1	Fillet 2	Fillet 3
Control (no dip)	1,320,000	770,000	2,670,000
Dip in .3% sodium benzoate	330,000	250,000	340,000
Dip in .15% sodium benzoate and .15% benzoic acid (total .3%)	140,000	180,000	220,000
Bacteria per gram after thawing and storing 24 hours at 3.3°C.(38°F.)			
Control (not dipped)	1,860,000	1,880,000	1,920,000
Sodium benzoate, .3%	520,000	570,000	620,000
Sodium benzoate, .15% and benzoic acid, .15%.	210,000	220,000	300,000
Bacteria per gram after thawing and storing five days at 3.3°C.(38°F.)			
Control (not dipped)	2,328,000	2,236,000	.....
Sodium benzoate, .3%	980,000	864,000	.....
Sodium benzoate, .15% and benzoic acid, .15%.	300,000	324,000	.....
Samples of frozen fillets after thawing and storing for 10 days. Bacteriological counts not made.			
Control (not dipped)	Spoiled and unfit for food.		
Sodium benzoate, .3%	Very faint odor. Spoilage barely perceptible.		
Sodium benzoate, .15% and benzoic acid, .15%	Good; no off-odor whatever.		

[1]A.P.H.A. standard agar; plates incubated at 32.2°C.(90°F.) for 48 hours.

TABLE 6

Effect of Benzoate Treatment on Keeping Quality and Bacterial Count[1] *of Defrosted Cod Fillets at 3.3°C.(38°F.)*

Treatment	Frozen		24 hrs. after defrosting		5 days after defrosting		10 days after defrosting
	Quality	Bacteria per gram	Quality	Bacteria per gram	Quality	Bacteria per gram	Quality
Control (not dipped)..	Excellent	1,580,000	Excellent	1,880,000	Stale	2,280,000	Putrid
Sodium benzoate, .3% in 5% brine.....	Excellent	307,000	Excellent	570,000	Good	922,000	Stale[2]
Sodium benzoate, .15% and benzoic acid, .15% in 5% brine.....	Excellent	180,000	Excellent	210,000	Good	312,000	Fair (edible)[2]

[1]All counts are the average of three fillets. [2]The samples were fried and tasted; while not of first quality they were classed as "edible" by three of the five judges. The odor was "strong fishy," not a putrefactive odor.

benzoate was slightly superior to the straight benzoate in preserving effect but also apparently left more residue in the fish.

Similar tests were conducted with red perch (rose fish) fillets. The fillets of this fish are small and relatively tender. Almost exactly similar results as with cod were obtained. Again there was no spoilage after five days' storage at 38°F., while the undipped controls were unfit for food. No flavor of benzoate was observed by any examiner except one, and that was very slight in the case of the benzoic acid-sodium mixture (.3 per cent total). The cooking test made after 12 days showed that both the benzoate treatments perfectly preserved the red perch fillets. After 19 days' storage both the cod and red perch fillets treated with .3 per cent sodium benzoate alone had partly spoiled, but those treated with the benzoate mixture (.15 per cent each of sodium benzoate and benzoic acid) were still edible but would be classed as "stale." Parenthetically, it should be mentioned that a considerable portion of the iced fish marketed in inland cities is far from fresh, and while not actually spoiled, it has a strong fishy taste and odor owing to trimethylamine and is quite unlike fresh fish.

RETENTION AND PENETRATION OF BENZOATES IN FILLETS DIPPED IN BENZOATED BRINES

In order to ascertain approximately how much benzoate actually remained in the flesh of haddock fillets dipped in benzoated brine, five experiments were made as follows:

The five-per cent salt brine contained in solution .2 per cent sodium benzoate. The dips were made in duplicate for two and 10 minutes and drained for one minute before weighing. Fillets were carefully weighed before dipping and after draining. The difference in weight was assumed to be absorbed water, salt, and benzoate. The calculation of benzoate retained was made on the assumption that there was no selective absorption of either sodium chloride or sodium benzoate.

The two-minute dip gave an average retention of .006 gram of sodium benzoate or .0054 per cent of benzoic acid per 100 grams of fish. The 10-minute dip gave an average retention of .011 per cent sodium benzoate or .009 per cent benzoic acid. Thus retention is too small to be of any health significance.

Chemical analysis of frozen haddock fillets which had been previously dipped in a brine containing .3 per cent sodium benzoate for five minutes gave values between .02 and .009 per cent.

Experiments were then made on haddock fillets dipped for two- and 10-minute periods in five-per cent salt brine containing .5 per

cent sodium benzoate. After periods of storage of 3, 24, and 120 hours, thin layers approximately one-eighth inch thick were cut from the fillet and examined for benzoate penetration by the qualitative method of Kolmer and Boerner (1938). While this color test is not entirely satisfactory, the results show that approximately three times as much salt and benzoic acid are found in the surface one-eighth inch as in the layer beneath, with only traces in the third one-eighth inch layer.

In a two-minute dip using .5 per cent sodium benzoate and five-per cent salt brine, the percentage of benzoic acid retained by haddock fillets was only .02 per cent.

It was proved by bacteriological study that the surface of fresh fillets contains nearly all the bacteria present. Very few organisms were found in the deeper layers of haddock or cod fillets except when they were stale. Because of fissures and breaks in the flesh, penetration of brines is uneven, at best. Attempts were made to ascertain the depth of penetration of brine and benzoate by means of eosin and erythrosin dyes. The results were not conclusive but showed that even a 10-minute dip in five-per cent salt brine penetrated only the surface layers of flesh except where these were broken.

TASTE THRESHOLD OF BENZOATES IN HADDOCK

Since it would avail nothing to use benzoic acid or its salts in the dipping solution if the fish were thus rendered unpalatable, a series of tests were made as follows:

Pieces of fillets were dipped in aqueous and brine (five per cent) solutions of sodium benzoate at .1, .2, .3, and .5 per cent levels for two- and 10-minute periods. The pieces were stored separately in the refrigerator at 2.2 to 4.4°C.(36 to 40°F.) for 48 hours, then removed and cooked for a 15 to 20-minute period in water. Several persons tasted these pieces of fillets for any trace of the characteristic benzoate taste. There was practical unanimity in the results. It was found that without brine, .3 and .5 per cent of sodium benzoate could be detected in fillets dipped for two and 10 minutes. However, the pieces immersed for two and 10 minutes with the five-per cent brine had no off-flavor or taste of benzoate, while those immersed for two and 10 minutes in .1 and .3 per cent of sodium benzoate alone, gave variable results with traces of the benzoate taste appearing in some instances. All pieces of fillets used in this test were stored for two weeks at 36 to 40°F. and none showed spoilage.

Essentially no differences were observed in the taste thresholds in haddock between sodium and magnesium benzoates or mixtures of these salts and benzoic acid. No advantage was found in dipping

the fillets first in the five-per cent salt brine and then in the benzoate solution, though the fish seemed to absorb more of the benzoate by this method. The sodium and magnesium benzoates used in these experiments had pH values of 7.4 and 7.3, respectively, in five-per cent salt brine. As a result of repeated experiments with as much as .3 and .4 per cent of benzoate salts in sodium chloride brine it would seem that no benzoate flavor will appear in cooked fillets of haddock and cod. The taste threshold of benzoic acid in cooked haddock was found to be about .07 per cent. Ordinarily much less than this amount remained in the flesh after dipping, draining, and packaging.

BENZOATE ICE

Briefly this investigation showed that it was entirely practical to freeze as much as .5 per cent of sodium benzoate in ice cakes under commercial conditions.[3] The concentration of benzoate in different parts of the ice blocks was determined. In general, it is most concentrated near the central and lower parts of the ice cake, with the lowest concentration at the periphery of the cake. The most feasible method of using benzoated ice, therefore, is to use it in crushed form.

Extensive experiments on round fish and fillets packed in benzoate ice containing from .15 to .30 per cent sodium benzoate, or mixtures of sodium or magnesium benzoate and benzoic acid, maintained the quality of the fish for several days longer than the controls held in ordinary ice. Marked reductions in the numbers of bacteria present were also noted. The pH of the ice had to be kept above 4 in order to prevent protein coagulation or "whitening" of the surface layers. However, benzoates were more effective when acidulated with hydrochloric or phosphoric acids.

There was some indication that the benzoate was selective in its action on bacteria. In some cases the bacteria were not greatly reduced over controls, yet the fish appeared definitely better. This observation checks that of Tarr and Sunderland (1938).

The taste threshold in haddock which had been iced in a .3 benzoated ice for five days and fried was approximately .07 per cent acid present in the flesh. Ice containing .5 per cent sodium benzoate produced a distinct flavor in the cooked fillets. Ordinarily, much less than this remains in the flesh after dipping in brine and momentarily draining. Care must be exercised, however, in using benzoated ice which remains in close contact with the fish for several days so that the flesh does not absorb excessive quantities of the benzoate. It

[3] The authors are indebted to Philip N. Simon of this laboratory who conducted the experiments on benzoate ice.

seems probable that the permissive concentration of benzoate in ice to be used for fish should be from .10 to .25 per cent.

There may be practical uses for benzoated ice, such as on trawlers, in storage, and while making shipments of round fish. Owing to benzoate absorption it seems unlikely that the ice will prove successful in the icing of fillets.

GENERAL CONCLUSIONS

Benzoates are useful in the fish industry for treating fillets and possibly round fish to extend their keeping quality. The recommended benzoates are sodium and magnesium and benzoic acid-benzoate mixtures. The bactericidal action of benzoates is increased by the addition of acids or by using benzoate salt-benzoic acid mixtures. These mixtures are very effective at pH values of from 4 to 4.5. Calcium and ammonium benzoates do not seem to be as effective as the others. Suggested salt concentrations for benzoates in dipping solutions are from .15 to .5 with perhaps .3 per cent as an optimum. The benzoated brine also has a cleansing and firming effect. The salt distinctly improves both flavor and texture of fillets. The action of the dipping solutions appears to be limited largely to the surface of the flesh. There is only slight penetration of benzoates in the deeper flesh layers except at points where the flesh has been broken.

In general, a two-minute dip is satisfactory.

The use of benzoate dips prolongs the storage life of haddock, cod, and red perch fillets for several days beyond that of untreated controls. This procedure should be of distinct advantage in preparing fillets for shipment. Frozen fillets, which have been previously treated with a benzoated brine, also keep longer after defrosting.

The retention of benzoates in brine-dipped fillets is very small, averaging about .01 per cent for a two-minute dip in a brine containing .3 per cent sodium benzoate.

The treatment of fish fillets with benzoates is a simple and practical way of extending their storage life without affecting their palatability or nutritive value.

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CHROMATOGRAPHIC IDENTIFICATION AND BIOLOGICAL EVALUATION OF CAROTENE FROM MATURE SOYBEANS

WILLIAM C. SHERMAN
Laboratory of Animal Nutrition, Alabama Polytechnic Institute, Auburn, Alabama

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The total carotene content of different varieties of soybeans in the green and mature stages has been reported from this laboratory by Sherman and Salmon (1939). The usual method of separating xanthophylls, chlorophyllines, and flavones from the carotene by means of a diphasic fractionation between aqueous alcohol and a hydrocarbon was employed. The carotene would, therefore, represent a mixture of various isomeric forms of carotene if these were present in the original material extracted.

Although fresh green plant material has been shown by Kuhn and Lederer (1931), Karrer and Schlientz (1934), and Hartman, Kane, and Shinn (1934) to yield carotene preparations which are nearly pure beta carotene, the nature of the pigments in carotene preparations from mature seed other than wheat and yellow corn has apparently not been investigated. Markley and Bailey (1935) have shown that the pigment remaining in the petroleum ether fraction from wheat is largely carotene. It has been reported by Kuhn and Grundmann (1934) and Fraps and Kemmerer (1937) that the "carotene" preparations from yellow corn, however, may contain 40 to 93 per cent of cryptoxanthin which has only one-half the biological value of beta carotene.

The identification of pigments in the carotene fraction from soybeans, therefore, seemed to be essential for the accurate estimation of the biological value of the carotene fraction. This fraction from five varieties of mature soybeans representing different color types and having a relatively high carotene content was subjected to chromatographic analysis. Samples from the same lots of soybeans were also fed to vitamin A-deficient rats as the sole source of vitamin A; the growth of these rats was compared with that of control rats receiving beta carotene or yellow corn at equivalent total "carotene" levels.

EXPERIMENTAL PROCEDURE

The varieties of soybeans used in these studies, with the exception of F.P.I. No. 93057, were grown locally on sandy-loam soil fertilized with superphosphate (400 lb. per acre) and muriate of potash (100 lb. per acre). The mature seed was stored at room tempera-

ture in covered galvanized cans for six months before the analyses and biological assays were made. There was not a sufficient supply of locally grown F.P.I. No. 93057 variety available for these studies. The seed of this variety was obtained from W. J. Morse of the United States Department of Agriculture. The green pigmentation of this shipment of seed was not as bright as of that grown locally and the carotene content was somewhat less.

Preparation of Samples for Chromatographic Analysis: For the chromatographic analyses a sample of each variety of soybeans was ground in an electric laboratory mill until it passed through a 20-mesh screen. Each sample (200 gm.) was divided into three parts, placed in Soxhlet extractors, and extracted first for six hours with methanol (synthetic), then for one hour with 95 per cent ethyl alcohol (purified by distillation after standing for several days with KOH), and finally with skellysolve [Skellysolve B, redistilled, boiling point 68-70°C.(154.4-158°F.)] for four hours. The combined extracts were concentrated to a volume of about 300 ml. on a boiling water bath and then saponified by boiling for one-half hour with 50 grams of KOH. After saponification, the solution was transferred to a 500-ml. separatory funnel. The usual method of extraction of xanthophyll and purification of the carotene solution—Sherman and Salmon (1939)—was employed. The purified carotene solution was concentrated under reduced pressure to about two to five ml. and subjected to chromatographic analysis with Tswett columns.

Method of Analysis: The method of chromatographic analysis described by Strain (1934) was applied on a micro scale to the purified extract from each variety of soybean. A small chromatograph tube, 11 x 140 mm., was used for the adsorption. It was uniformly filled with an intimate mixture of equal parts by weight of magnesium oxide (Micron Brand, 2641, manufactured by the California Chemical Company, Newark, California) and heated siliceous earth (Hypho Super Cel, manufactured by Johns Manville). The tube was fitted into a rubber stopper and placed into a suction flask. Skellysolve was forced through the tube until the entire column was covered with solvent. The concentrated extract from the soybeans contained in skellysolve solution was then poured into the tube and washed with fresh skellysolve until the bands, usually three in number, were well separated.

The procedure generally followed in separating the pigments was to continue washing with skellysolve until the band of least adsorbed pigment had been washed to the bottom of the tube where it was collected in a test tube. The band of most highly adsorbed pigment was scraped from the top of the column into a small beaker

containing skellysolve with added methanol. In most instances only one band was left on the column; it contained the major portion of the pigment. This pigment was eluted by passing a solution of equal parts by volume of skellysolve and methanol through the tube, collected in a test tube, and transferred to a small separatory funnel. The solution of the pigment scraped from the top of the adsorption tube was decanted from the adsorbent into another separatory funnel. The pigment was completely removed from the adsorbent remaining in the beaker by washing two or more times with skellysolve containing methanol and transferred to the separatory funnel. The methanol was removed from the solutions in the separatory funnels by washing three or four times with distilled water. The skellysolve solutions of the pigments were then dried with anhydrous Na_2SO_4 and made to suitable volume for the determination of pigment content.

To determine the relative amounts of the different pigments, the skellysolve solutions were read on a universal spectrophotometer at a wave length of 475 mμ. The pigment content of the solutions was calculated from the equation $c = D/kl$, where c is the concentration of pigment in grams per liter of solution, D is the optical density as read directly from the spectrophotometer, l is the length in centimeters of the layer of solution, and k is the specific absorption coefficient for the pigment. The value 229, obtained for the specific absorption coefficient at 475 mμ of a skellysolve solution of beta carotene (S.M.A. beta carotene recrystallized from petroleum ether-mentanol 1:2) was used for k in calculating the concentration of all pigments. A slight error is thus introduced in the calculations for pigments other than beta carotene, but the specific absorption coefficient for cryptoxanthin in skellysolve has not been determined. Buxton (1939) has recently reported a value of 243 for the specific absorption coefficient at 452 mμ of purified cryptoxanthin in heptane. A value of 223.5 has been published by Shrewsbury, Kraybill, and Withrow (1938) for the specific absorption coefficient of alpha carotene at 475 mμ in heptane.

After the quantity of each pigment had been determined, the solutions were evaporated to small volume (1-3 ml.) for identification. An equal amount of known pigment contained in a small volume of skellysolve was thoroughly mixed with the unknown pigment and passed through a 7 x 100 mm. chromatograph tube packed with magnesium oxide-siliceous earth. The appearance of a single band established the identity of the pigment.

The known pigments used in identification were alpha and beta carotene and cryptoxanthin. The alpha and beta carotene were pre-

pared from crystalline carotene (S.M.A. Corporation, mfg. spec., 90 per cent beta and 10 per cent alpha carotene). The crystalline carotene was dissolved in skellysolve and passed through a magnesium oxide column of suitable dimensions to effect a separation of the alpha and beta bands. The two bands were eluted, washed, and dried with anhydrous Na_2SO_4. The skellysolve solutions of alpha and beta carotene were each passed through another adsorption tube for further purification and were stored in the dark at 3°C.(37.4°F.) for reference in chromatographic work. The cryptoxanthin used in chromatographic identification was prepared from yellow corn. The method of extraction and the subsequent fractionation of the corn pigments with methyl alcohol and skellysolve were the same as described above for soybeans. The purified skellysolve fraction from yellow corn was passed through an 11 x 140 mm. adsorption column packed in the usual manner. The column was washed with fresh skellysolve until the three least adsorbed bands containing alpha, beta, and gamma carotene were completely removed at the bottom. The cryptoxanthin was eluted from the column with skellysolve-methanol (1:1). The solution was washed with water in a separatory funnel, dried with Na_2SO_4 and stored in the refrigerator. Spectrophotometric readings made on the solutions of cryptoxanthin and combined carotene showed that the purified extract from the yellow corn contained 66 per cent cryptoxanthin and 34 per cent mixed carotenes.

Method of Biological Assay: The rats used in vitamin A assays were taken from stock females (Wisconsin strain) which were fed a diet low in vitamin A while nursing the litters. The day after the litters were born the females were placed on the modified stock diet and maintained upon it until the litters were weaned. For this purpose the regular stock diet used in this laboratory—Prickett (1934)—was modified by the omission of alfalfa meal, cod liver oil, and butter fat, making an equivalent increase of eight per cent in the content of whole wheat. During this period the females also received a dry supplement consisting of three parts skim milk powder and one part coconut fat, which was fed *ad libitum* instead of whole milk. After the litters were weaned the stock females were again placed on the regular stock diet supplemented with whole milk.

When the young rats (usually eight to the litter) reached a weight of 40-50 grams (23-26 days) they were transferred to individual metal cages and fed the vitamin A-test diet 31-A[1] and distilled water

[1] The vitamin A-test diet consisted of casein (purified by alcoholic extraction) 18; salt mixture No. 186 (J. Biol. Chem., **89**, 199, 1930) 4; Northwestern dried yeast, irradiated 6; and sucrose 72.

ad libitum. All rats received a daily supplement of .1 ml. of decolorized soybean oil [2] throughout the depletion period to supply essential fat constituents. The use of oil from varieties of soybeans which were low in carotene and the subsequent decolorization of the oil with fuller's earth yielded a product in which the carotene was so low that the addition of .1 ml. daily had no effect upon the length of depletion period of the rats. The rats were weighed weekly during the first four weeks of the depletion period, after which they were weighed daily until growth had ceased and ophthalmia had appeared (usually by the end of the fifth week of depletion). The depleted animals were then fed the supplements daily and weighed weekly throughout the test period. Sex distribution was practiced, and at least two rats of each litter were fed either beta carotene or yellow corn for positive controls. Negative controls were also used at frequent intervals. The feeding of soybean oil (.1 ml. daily) to rats receiving carotene or yellow corn was continued during the test period but was discontinued in the group receiving soybeans.

The yellow corn and the varieties of soybeans to be assayed for vitamin A were ground and stored in covered glass jars in a dark, cold-storage room at 3° C. (37.4° F.). Once each week one week's requirement of the soybeans and corn was removed from the refrigerator and kept at room temperature in covered jars for feeding. The soybeans and yellow corn were fed on the basis of the total carotene content which was determined shortly before assays were begun according to a method previously described by Sherman and Salmon (1939).

The carotene used in feeding experiments was S.M.A. beta carotene. The crystals were dissolved in skellysolve and diluted to suitable volume so that two gamma of carotene, the usual daily dose, were contained in .1-.2 ml. volume. Carotene solutions were stored in the dark at 3°C. The exact concentration of carotene in the solutions was determined by spectrophotometric reading before they were fed and at bi-weekly intervals thereafter in order to make any necessary changes in the aliquots for feeding. It was found that the destruction of beta carotene at this concentration in skellysolve solutions stored in the refrigerator was only about .5 per cent in 30 days.[3] In feeding, the aliquots from the carotene solutions were pipetted each morning on about three-gram quantities of the basal diet con-

[2] The soybean oil was extracted from a pale yellow variety of soybeans and was decolorized by passage through a column packed with fuller's earth.

[3] More concentrated solutions of several samples of S.M.A. crystalline carotene in skellysolve (400-500 gamma per ml.) showed from 11 to 40 per cent destruction in 30 days' storage at 3°C.(37.4°F.).

tained in separate feed jars. When the skellysolve had evaporated, the contents of the jar were mixed by spatula, and .1 ml. of decolorized soybean oil was then added. The jars containing the supplements were immediately placed in the individual cages and the contents were usually completely consumed within 15 minutes. The possibility of carotene destruction in this method of feeding was investigated. A solution of S.M.A. beta carotene in skellysolve was added by pipette to a three-gram portion of the basal diet and allowed to stand at room temperature in an open feed cup for 45 minutes after the solvent had evaporated. The carotene was then extracted with skellysolve and a spectrophotometric reading made. Over 99 per cent of the original carotene was recovered.

RESULTS

Chromatographic Analyses: The purified extracts from the following varieties of mature soybeans were subjected to chromatographic analysis: F.P.I. Nos. 85384, 93057, 94168, Matthews, and Kura. They represent a fairly wide range in color types. The seed coats of the different varieties were bright yellow, bright green, olive-brown, black, and mottled (black and pale green), respectively; the cotyledons of all varieties were bright yellow with the exception of F.P.I. No. 93057, which were bright green in color.

When passed through adsorption tubes the purified extracts from all five varieties of soybeans gave the same general type of adsorption. Upon washing, the pigment on the column separated into three distinct bands. A narrow band of alpha carotene passed through first and was followed by a wide band of beta carotene which contained most of the total pigment. A portion of the pigment remained strongly adsorbed to the two to three millimeters at the top of the column. This strongly adsorbed pigment will be designated as residual pigment in the following pages. A very faint band of pigment usually appeared between the residual and beta carotene bands, but in all varieties except Matthews this band was so diluted in the washing, which was necessary to effect a good separation of alpha and beta carotene, that it had disappeared when the washing was completed. In the Matthews variety this band was still present after washing; it was separated and eluted from the adsorbent. Spectrophotometric reading of a skellysolve solution of this pigment showed that it comprised only 1.4 per cent of the total pigment eluted from the column. Although there was an insufficient amount of pigment in the band to permit identification, its relationship to beta carotene, being somewhat more strongly adsorbed than the latter, would indicate that the pigment was gamma carotene.

The amount of pigment in each of the three main bands formed when the purified extracts from the five varieties of mature soybeans were passed through chromatograph tubes, together with the total carotene content, is given (Table 1). It was found that most of the pigment (80-88.8 per cent) in the purified extracts was beta carotene. The beta carotene was identified in each variety by mixing it with an equal amount of known beta carotene and passing the solution through another chromatograph tube. In each case all of the pigment remained in a single band (beta carotene).

The relative amount of alpha carotene obtained from the different varieties was in greater variation, but in each the alpha carotene comprised a very small portion of the total pigment (2.5-11.8 per cent of the total). The alpha carotene was identified by mixing it

TABLE 1

Chromatographic Separation of Carotene of Soybean Seed

Variety	Total pigment	Alpha carotene	Beta carotene	Residual pigment
	gamma /100 gm.	*pct.*	*pct.*	*pct.*
F.P.I. No. 85384	112	9.2	81.0	9.8
F.P.I. No. 93057	174	11.8	80.0	8.2
F.P.I. No. 94168	158	4.2	87.5	8.3
Matthews[1]	117	2.5	88.8	7.3
Kura	92	6.1	87.6	6.5

[1]Another band representing 1.4 per cent of the total pigment, apparently gamma carotene, was separated.

with an equal amount of known alpha carotene and passing the solution through a small chromatograph tube (7 x 100 mm.). With each variety the mixed solutions reacted as a single compound.

In attempting to identify the residual pigment, it was found that the strongly adsorbed band was a mixture of compounds. The residual fraction from the Matthews variety was mixed with cryptoxanthin and passed through a small chromatograph tube filled with a less active preparation of magnesium oxide (Merck, U.S.P. heavy). The cryptoxanthin was only slightly adsorbed and passed through the column rapidly. The residual pigment was loosely adsorbed within the top half of the column, but the brand of magnesium oxide used was too inert to give distinct band formation. The residual fraction from Kura soybeans was poured into a 7 x 100 mm. chromatograph tube filled with the usual 50-per cent mixture of micron brand magnesium oxide. The pigment separated into three faint but distinct bands after extended washing. Because of the small amount of pigment in bands from the residual fraction of the soybeans, they were not further identified.

TABLE 2

Gains of Rats Receiving Mature Soybeans, Yellow Corn, and Beta Carotene as Sources of Vitamin A

Supplement [1]	Carotene [2] content	Number of rats	Average gain in weight at end of						
			1st week	2nd week	3rd week	4th week	5th week	6th week	7th week
	gamma /100 gm.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
F.P.I. No. 93057 soybean (1.15 gm.)	174.0	9	13 (—1-26)	33 (2-46)	48 (33-62)	62 (43-88)	72 (52-107)	80 (60-112)	85 (61-123)
Kura soybean (2.17 gm.)	92.1	4	17 (7-21)	31 (24-36)	51 (44-57)	63 (49-69)	68 (49-79)	74 (50-87)	79 (50-94)
F.P.I. No. 85384 soybean (1.79 gm.)	112.0	3	15 (14-15)	32 (27-36)	48 (37-54)	58 (37-74)	62 (37-84)	74 (55-93)	81 (68-93)
Matthews soybean [3] (1.71 gm.)	117.0	3	12 (7-14)	22 (19-23)	41 (36-51)	46 (44-51)	47 (44-51)	49 (44-53)	51 (44-57)
F.P.I. No. 94168 soybean [3] (1.27 gm.)	158.0	4	6 (4-12)	16 (11-24)	16 (11-21)	19 (11-26)	21 (11-33)	23 (11-42)	23 (11-42)
Yellow corn (.33 gm.)	603.0	15	5 (—9-13)	16 (4-23)	25 (9-49)	35 (25-65)	42 (26-75)	49 (33-83)	54 (27-93)
Beta carotene (2 gamma)	.....	4	10 (7-12)	23 (14-27)	34 (32-36)	48 (44-52)	62 (49-69)	74 (64-81)	86 (82-94)

[1] Each supplement was fed in an amount sufficient to supply two gamma of total carotene daily. [2] Carotene refers to the total pigment determined in the purified skellysolve extracts. [3] Matthews and F.P.I. No. 94168 varieties of soybeans were unpalatable to the rats and were not completely consumed.

Biological Assays: The summarized results from the biological assays (Table 2) show that the average gain in weight obtained when F.P.I. Nos. 93057, 85384, and Kura varieties of soybeans were fed on an equivalent carotene basis (two gamma daily) to vitamin A-deficient rats was not significantly less than that obtained from two gamma of beta carotene. Yellow corn, however, gave considerably less gain in weight although it was fed at a level to supply the same amount of total "carotene." This is to be expected since chromatographic analyses showed that 66 per cent of the total pigment of the extracts from yellow corn was cryptoxanthin.

The inferior growth obtained when two varieties of soybeans (Matthews and F.P.I. No. 94168) were fed was a result of incomplete consumption of supplements. These two varieties were bitter in flavor and were only partially consumed by the rats after about the second week. The amount consumed was insufficient to meet the requirements for vitamin A, and the animals which survived the test period exhibited ophthalmia at the end of the experiment.

The results from the bio-assays confirm the chromatographic analyses in showing that the vitamin A potency of mature soybeans, in contrast with yellow corn, results from the beta carotene. The carotene content of many varieties of soybeans at maturity is as great as that of yellow corn. In terms of total vitamin A activity, however, the yellow corn, because of the presence of relatively large amounts of cryptoxanthin, was superior to all the varieties of mature soybeans studied. The superiority of yellow corn to soybeans as a source of vitamin A has been reported by Mitchell and Beadles (1936).

SUMMARY

Chromatographic analyses of the biologically active carotinoid pigments extracted from the mature seed of five varieties of soybeans demonstrated that the pigment contained in the purified extracts was 80-88.8 per cent beta carotene and 2.5-11.8 per cent alpha carotene. Cryptoxanthin was not found in any of the soybeans analyzed. Similar extracts of yellow corn were found to contain 66 per cent cryptoxanthin. The results from the chemical analyses were substantiated by biological assays.

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EFFECTS OF CONTINUED FEEDING OF DIPHENYLENE OXIDE[1]

JOHN O. THOMAS, ROBERT H. WILSON, AND C. W. EDDY

Bureau of Agricultural Chemistry and Engineering, United States Department of Agriculture, at the Department of Pharmacology, Stanford University School of Medicine, San Francisco, California

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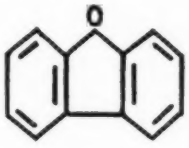
Among the organic compounds which have received attention from the viewpoint of insecticidal action, and which have been investigated in this laboratory for evidence of potential chronic toxicity to mammals, is diphenylene oxide. This compound has a disagreeable odor and taste, is practically insoluble in water, melts at 81°C.(177.8°F.), boils at 288°C.(550.4°F.), and has the following structural formula:



The immediate problem was to determine whether or not spray residues, which contain diphenylene oxide following its use as an insecticide, might present a public-health hazard. Since any spray residue containing this substance would necessarily gain entrance to the body by absorption from the gastrointestinal tract, the possibility of producing symptoms of chronic poisoning was investigated by incorporating the compound in the basic ration routinely used in this laboratory, and feeding it to albino rats. The ration consisted of the following:

Corn meal	73.0 per cent
Linseed oil cake	10.0 per cent
Alfalfa	2.0 per cent
Casein	10.0 per cent
Cod liver oil	3.0 per cent
Bone ash	1.5 per cent
Sodium chloride	0.5 per cent.

A group of five female rats, about 30 days old, was used as controls and fed the unmedicated diet. Groups of rats of the same number, sex, and approximately the same age were placed in sep-

[1] Food Research Division Contribution No. 434.

arate cages and fed the same diet thoroughly mixed with various concentrations of diphenylene oxide. The concentrations used were .025, .05, .1, .2, and .4 per cent, corresponding to 250, 500, 1,000, 2,000, and 4,000 parts per million, respectively. All cages were provided with water bottles which permitted free access to water at all times, and with food cups designed to reduce food losses to a minimum. The individual rats were weighed twice a week and

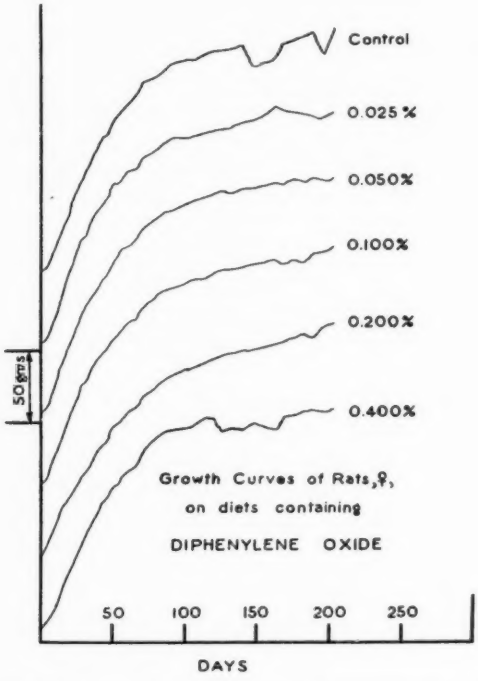


FIG. 1. Growth of young rats fed increasing concentrations of diphenylene oxide in the diet.

growth curves plotted. Food cups were weighed at the same time to obtain a record of the food intake. Observations were made on general behavior, blood picture, water intake, and urine output. After the feeding experiments were terminated all rats were killed, their body lengths measured, and their organs weighed according to the autopsy procedure used in this laboratory by Wilson, McNaught, and DeEds (1938). Histological examination of tissue sections was made for evidences of pathological changes.

The low solubility of diphenylene oxide in water might theoretically suggest that absorption from the gastrointestinal tract would be limited and the possibility of poisoning remote. However, it is never safe to apply such a generalization to a specific instance. Low water solubility *in vitro* does not necessarily mean low solubility under the conditions in the gastrointestinal tract. Even low solubility in the digestive tract does not necessarily preclude systemic absorption. For instance, phenothiazine is practically insoluble in water, but the oral administration of this compound to humans is followed by the appearance of its oxidation products—thionol and leuco thionol—in the urine within 30 minutes, often within 15 minutes, and the peak of excretion is reached in six to 12 hours, varying somewhat from individual to individual and with conditions in the digestive tract (DeEds, Eddy, and Thomas [1938]). With other agents the absorption may be slight but the toxicity so great that the meager amounts absorbed will suffice to produce toxic symptoms. This may be especially true in chronic intoxication when there is continuous subjection of the tissues to the action of low concentrations of poison for long periods. Cryolite (Na_3AlF_6) well illustrates this process. The physiological effects of fluorine, according to DeEds and Thomas (1934), are sufficiently great to demonstrate that the amount of fluorine absorbed when fed in the form of the poorly soluble cryolite is practically as effective as fluorine in the form of sodium fluoride.

EFFECTS ON GROWTH

The growth curves of the control rats and of those on diets containing diphenylene oxide (Fig. 1) show that the diphenylene oxide exerted so slight an effect on the growth rates based on body weight that the growth curves are separated to prevent superposition. Since a concentration as high as 4,000 parts per million in the diet produced no retardation of growth the experiment was repeated on another group of rats (Fig. 2).

Again the diphenylene oxide caused no demonstrable retardation of growth. The general shape of the growth curves, including the control, is somewhat flatter than that in Fig. 1, but this difference probably reflects the effects of the season and of general environmental conditions.

In view of the failure to demonstrate retardation of growth on diets containing .4 per cent diphenylene oxide, two female rats weighing 186 and 190 grams were placed on 8,000 parts of diphenylene oxide per million parts of diet, but without demonstrable effects on growth (Fig. 2).

In harmony with the negative evidence on growth rates were the following facts: food intake throughout the experiment was not appreciably altered, and none of the animals exhibited any abnormalities of behavior or activity.

WATER BALANCE

Over a period of time an impression was gained that the water bottles had to be filled more frequently for the rats fed diphenylene oxide. To permit observation of changes in water balance, the following experiment was made on 10 rats. Five male rats averaging 255 grams and weighing a total of 1,273 grams were used as controls and placed in a cage provided with a food cup, a graduated water bottle, and a receptacle for collecting the urine. A similar group of five male rats averaging 255 grams and weighing a total of 1,274 grams was placed in a similar cage and given the same diet to which diphenylene oxide (.5 per cent or 5,000 parts per million) had been added. In order to avoid accidental errors in measurement of the apparent water consumption, the graduated water bottles were ex-

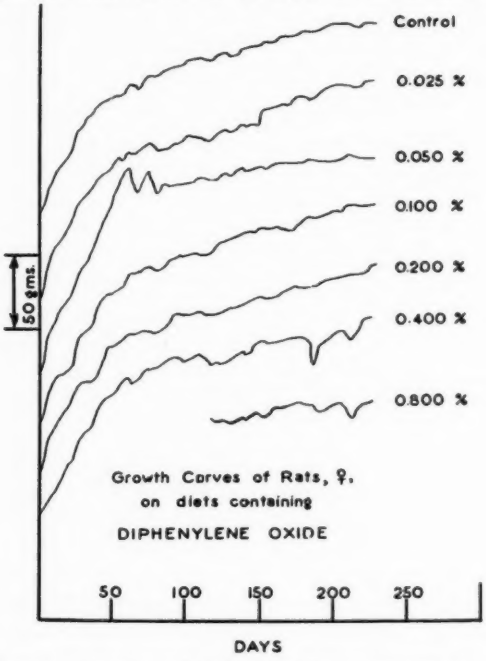


FIG. 2. Growth of young rats fed increasing concentrations of diphenylene oxide in the diet.

changed on the two cages on alternate days. Observations were made of water intake, urine output, food consumption, and body weight for a period of 78 days.

The results were as follows: The control group increased in weight from a total of 1,273 grams to 1,594 grams, or showed an increase of 321 grams, corresponding to an average increase in weight of 64 grams per rat. The rats receiving .5 per cent diphenylene oxide in the diet increased in weight from 1,274 grams to 1,517 grams, corresponding to an average gain in weight of 49 grams per rat. The control rats ate 6,108 grams of food as compared with 5,482 grams for the experimental animals, corresponding to an average excess in food consumption of 125 grams per rat for the former group. On the other hand, the control group drank 9,652 c.c. of water, and the group receiving diphenylene oxide 10,316 c.c., or an average excess of 111 c.c. of water per rat in favor of the latter group. In harmony with the increased water intake of the experimental animals, there was an increase in urine output over that of the control group. The control group excreted a total of 3,110 c.c., whereas the medicated group excreted 3,984 c.c., or an average excess of 146 c.c. of urine per rat. Thus, the control animals drank 6,542 c.c. more than they excreted in the urine, and the rats receiving diphenylene oxide, 6,332 c.c. more. The difference between this excess of water intake over urine output of the controls and the corresponding excess of the diphenylene-oxide rats, was 210 c.c., corresponding approximately to .5 c.c. daily per rat. It is doubtful that this could be regarded as a significant diuresis, or dehydration of the tissues, especially since an increase of 78 grams in weight of the whole control group as compared with the medicated group could well be accounted for by the excess of 626 grams of food consumed by the controls.

The data on body weights, food intake, water intake, and urine output for the entire period of 78 days are shown graphically (Fig. 3). In each case the data for the control group are shown by the solid lines and the corresponding values for the medicated group are indicated by crosses. It is evident that for short-time periods the same relationships hold in regard to weight, food intake, water intake, and urine output as stated above in summary for the entire period. It is also evident that the increased water intake and urine output were not appreciable until the experiment had been in progress about 15 days. This suggests that the effect of diphenylene oxide on water balance is of a chronic nature rather than an acute effect.

The absence of a significant dehydration in these adult rats is consistent with the absence of a significant retardation of weight increase (Figs. 1 and 2). It is evident, however, that the addition

of .5 per cent diphenylene oxide to the diet resulted in an increase of water intake and urine output. The experiment does not furnish evidence as to whether diphenylene oxide caused an increased urine output consequent to an increase in water intake, or vice versa.

BLOOD PICTURE

The rats, which had been on the diet containing .5 per cent diphenylene oxide for 78 days, were examined for possible hema-

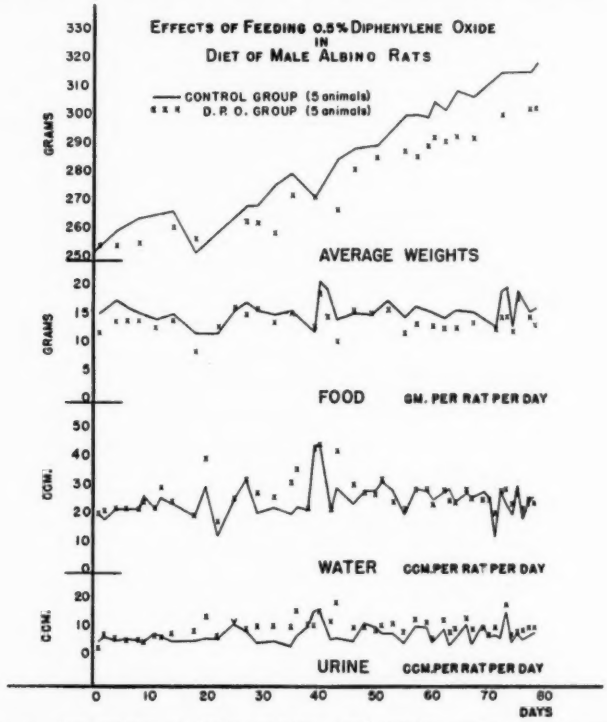


FIG. 3. Comparison of body weights, food intake, water intake, and urine output of control group of rats and group fed .5 per cent diphenylene oxide in addition to regular diet.

toxic effects. Hemoglobin was determined in grams per cent, and counts were made of the erythrocytes, reticulocytes, and white cells; differential counts of the white cells were also made. The values for the five rats agreed well among themselves, the averages being as follows: 16.6 grams per cent hemoglobin, 9,070,000 erythrocytes, 2.35 per cent reticulocytes, and 16,480 white blood cells. The corresponding average values for 10 control rats of approximately the same

age and weight were 16.3, 8,120,000, 3.0, and 14,400, respectively. The differential white-cell counts of rats receiving diphenylene oxide gave average values as follows: lymphocytes 63.8, polymorphonuclear neutrophiles 33.5, monocytes 1.18, basophiles .64, and eosinophiles .94 per cent. The corresponding values for normal rat blood reported by Scarborough (1930-31) are 67.9, 27.0, 5.3 (range 2 to 7 per cent), .77, and 2.1 per cent, respectively. Accordingly, the ingestion of food containing .5 per cent diphenylene oxide over a period of 78 days, during which time each rat ingested an average of 5.5 grams of the compound, produced no demonstrable changes in the blood.

AUTOPSY FINDINGS

At the conclusion of the experiment on growth rates, the control rats and those receiving .025, .05, .1, .2, and .4 per cent diphenylene oxide were sacrificed. The gross appearance of all animals was normal, except that all the rats receiving diphenylene oxide showed unusually large amounts of abdominal fat. A comparison of body lengths and organ weights showed all these measurements of the rats receiving diphenylene oxide to be appreciably less than those of an extensive series of control rats. Since this was true of all the measurements, and the growth curves showed practically no inhibition of weight increases, it is evident that the presence of abnormal amounts of abdominal fat introduced an error in growth curves based on body weight. This agrees with the statement of Hatai (1913) that body length is a more accurate criterion of growth than is body weight. However, the difficulties of making accurate body-length measurements on living rats are such that body weight was used as a matter of practical convenience.

HISTOLOGICAL FINDINGS

Histological examination of the livers, kidneys, spleens, hearts, and adrenals of rats on the dosage range from .05 to .8 per cent diphenylene oxide was made by Dr. Alvin J. Cox of the Department of Pathology of Stanford University. All sections were stained with hematoxylin and eosin. No significant abnormalities were seen except in the kidney sections. Dr. Cox's report is as follows:

"All specimens of kidney show accumulation of fine brown pigment granules in the epithelial cells of the proximal convoluted tubules lying in the deeper parts of the renal cortex. The amount of pigment bears a direct relationship to the dose of diphenylene oxide. In animals receiving the larger doses, the pigment granules are very closely placed within the cells, and nearly every cell of an affected convoluted tubule is involved. With the smaller doses, the change is slight and much less uniform, some cells containing numerous granules, while adjacent cells contain few or none. There is curious localization of the pig-

mentation to the cells of the tubules lying in the deeper portions of the cortex; those at the periphery show no pigmentation even with the larger dose. In no case is pigment found in the cells of Henle's loops, the distal convoluted tubules, or the collecting tubules.

"The pigment does not give the Prussian blue reaction, nor does it stain with Sudan III to an appreciable extent. It resembles the pigment present in small amounts in the tubular cells sometimes seen in normal rats and demonstrable in a few scattered cells in 2 of the 5 control rats of this series. No further attempts at identification of the pigment were made.

"In the kidneys of the two rats on the diet containing 0.8 per cent diphenylene oxide, there is a prominent irregular dilatation of the collecting tubules, many of which contain coagulated material resembling protein. The other tubules are also slightly dilated in places and contain more granular and amorphous material than do the controls. There are no definite evidences of degeneration of the cells themselves, and no abnormalities of the glomeruli are seen with this stain. Some of the kidneys from animals fed 0.4 per cent diphenylene oxide show slight similar changes, but no significant deviations from normal in this respect are found in the animals fed smaller amounts.

"Several of the animals in the 0.8 per cent and 0.4 per cent groups show slight hyperplasia of the Malpighian bodies in the spleen, but no other abnormalities are noted. The livers, hearts, and adrenals of the experimental animals do not differ significantly from those of the controls."

The renal changes reported by Dr. Cox constitute the only evidence that irritation of the kidney may have been responsible for the increase in urine output in the experiment on water balance, and that the increased water intake might have been secondary and of a compensatory nature. On the other hand, the odor and taste of the diphenylene oxide, which was distinctly noticeable in the diet containing .5 per cent, may have led to an increase in water intake followed by an increase in urine output. The fact that the changes in water balance were not evident until the rats had been on the medicated diet about 15 days suggests that the first hypothesis is the more likely. Whatever the explanation of the "diuretic" phenomenon, it was unforseen at the outset of the experiment. It may be that as a result of this investigation there has been brought forth another manifestation, or criterion, of chronic physiological disturbance which will be valuable in studies on possible health hazards of chemical substances, contaminated diets, and the like.

SUMMARY AND CONCLUSIONS

1. Albino rats were fed a balanced diet containing diphenylene oxide in concentrations from .025 to .4 per cent for periods of 200 days.

2. No demonstrable effect on weight increment as compared with controls was noted.

3. Body lengths and organ weights were somewhat less in rats on diets containing diphenylene oxide than those of control rats of

comparable weights, but the amount of abdominal fat at autopsy was greater.

4. Urine output and water intake were increased by the continued feeding of a diet containing .5 per cent diphenylene oxide.

5. The blood picture remained normal.

6. Histological evidence of tissue damage was absent in the liver, spleen, heart, and adrenal but present in the kidney.

7. Since the minimal dose of diphenylene oxide administered corresponded to a concentration of 250 parts per million parts of diet, it seems unlikely that the use of diphenylene oxide as an insecticidal spray, would present a spray-residue problem, provided the diphenylene oxide is not changed to some more toxic compound by weathering or other conditions.

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CHANGES IN VITAMIN C CONTENT DURING BOILING OF TURNIP GREENS IN VARIOUS WATERS IN COVERED AND UNCOVERED CONTAINERS[1]

W. W. FLOYD
Sam Houston State Teachers College, Huntsville, Texas
AND
G. S. FRAPS
Texas A. & M. College, College Station, Texas

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In the southern states turnip greens are widely produced and used as a table food. They constitute a highly important source of vitamin C in the diet throughout much of the year. According to Tauber and Kleiner (1934) they contain 110 mg. of vitamin C per 100 grams of sample. Since they are rich in vitamin C and are grown almost continuously during all four seasons, turnip greens were selected for the present study.

Many articles have been published on the effect of boiling on the vitamin C content of vegetables. McHenry and Graham (1935) found that cooked peas had 42 per cent less vitamin C than raw peas. Vinokurov, Eidelman, and Buton (1935) called attention to the fact that much of the vitamin C goes into the cooking liquor upon boiling cabbage. Taking into account the vitamin C that dissolved in the cooking waters, Fenton, Tressler, and King (1936) found losses of seven to 10 per cent of the vitamin C when peas were boiled in sodium chloride solutions in an enamel pan. According to Dunker, Fellers, and Fitzgerald (1937), heating sweet corn destroys seven to 10 per cent of the vitamin C. Fenton, Tressler, Camp, and King (1937a) reported losses of five to 19 per cent in vitamin C during the cooking of Swiss chard in a salt solution in an enamel pan. Richardson, Davis, and Mayfield (1937), however, claimed that boiling potatoes 35 minutes at 95°C.(203°F.) caused no change, whereas baking them for one hour at 225°C.(437°F.) caused an increase in vitamin C content. Newton and Lowry (1937) reported that the amount of vitamin C was unchanged in Puerto Rico sweet potatoes boiled for 35 to 45 minutes. Wellington and Tressler (1937) reported that boiling cabbage destroyed less than one-sixth, steaming destroyed more, and frying destroyed about one-third of the vitamin C. Fenton, Tressler, Camp, and King (1937b)

[1] Presented in part at the Baltimore meeting of the American Chemical Society, April 5, 1939.

reported that about 11 per cent of the vitamin C was destroyed in the boiling of carrots. Fixsen and Roscoe (1938) and Fenton and Tressler (1938) report a loss of five to 15 per cent in boiling carrots, peas, and chard. Daniel and Munsell (1937) give extensive references to vitamin C.

EXPERIMENTAL PROCEDURE

This study was undertaken to determine the influence of the cooking water, the rate of boiling, the material of the cooking vessel, and the covering or not covering of the vessel on the changes produced in vitamin C content when boiling turnip greens. For our purpose turnip greens were boiled in distilled water, in sodium bicarbonate and sodium carbonate solutions, and in neutral and slightly acidic sodium acetate solutions. Sodium bicarbonate is found in appreciable quantities in some of the domestic waters of this section of the country. Covered and uncovered aluminum, porcelain enamel, stainless steel, and pyrex vessels were used. In the first series of experiments, 2.5 gm. of the greens to 50 c.c. of water were used and the reversibly oxidized ascorbic acid was not reduced. In the second series, 50 gm. of greens to 75 to 200 c.c. of water were used and the oxidized ascorbic acid was reduced. The latter more nearly resembles practical cooking conditions.

Samples for analysis were taken from one side of the midrib of fresh, mature turnip leaves possessing markedly perfect bilateral symmetry, and the sample for boiling was taken from the other side. The leaves were freed of any adhering droplets of water by blotting with filter paper immediately before weighing. No stems or midribs were used, since these possess very little of the vitamin. As in previous determinations of vitamin C in Texas fruits and vegetables—Floyd and Fraps (1939)—the method of Bessey and King and a modification of the extracting solution suggested by Dr. D. K. Tressler of New York State Agricultural Experiment Station were used, but with three successive extractions and filtrations instead of only two. The 2.5-gm. sample was extracted with 10, 12, and 14 ml. portions, successively, of 2% HPO_3-5%H_2SO_4 solution. The 50-gm. sample was extracted three times with 75-ml. portions of the acid. After thorough grinding with sand and acid, the sample was filtered through a Buechner funnel on paper for the 2.5-gm. sample, and on cloth for the 50-gm. sample. The residue and paper were taken for the re-extraction. The filtrate from the 2.5-gm. sample was collected in a 100-ml. volumetric flask and made up to volume with eight per cent acetic acid. After shaking the solution, five-ml. aliquots were titrated with a standardized solution of the sodium salt of 2,6-dichlorophenolindophenol with use of a microburette. A blank con-

taining only the acids was run. The dye solution was standardized against fresh ascorbic acid, the ascorbic acid against iodine, and the iodine against arsenious oxide from the U. S. Bureau of Standards.

The filtrate from the 50-gm. sample was reduced with hydrogen sulphide, allowed to stand 20 minutes, the excess hydrogen sulphide removed with carbon dioxide, made up to 500 ml. with eight per cent acetic acid, and five milliliters were titrated.

The treatment with hydrogen sulphide reduces reversibly oxidized ascorbic acid and this is biologically active.

Each sample for boiling was taken from the other side of the midrib of the leaves used in the analysis immediately preceding, midribs and stems being rejected. With the 2.5-gm. samples 50 ml. of water or solution was put into a half-pint cup, with or without a watch glass cover; brought to boiling by a small, reasonably constant Bunsen flame; and kept boiling two minutes before the sample was put in. The sample was tamped down below the liquid with a glass stirring rod, which was then removed. It usually took 20 seconds for the cooking water to resume boiling following the introduction of the sample. The flame used in all cases was one just sufficiently hot to keep the liquid boiling in the open cups. The greens were kept boiling for 15 minutes over the same small flame, then the cup was removed, plunged immediately into a large bath, and brought to the temperature of the tap water. If the cup was covered during boiling, it was likewise cooled with the cover on. The 30 or 35 ml. of supernatant cooking liquor was decanted into a 100-ml. volumetric flask, the liquid clinging to the leaves being separated by pressing with the stirring rod. The cup, cover (if any), and greens were washed with two two-ml. portions of distilled water and the washings added to the cooking liquor. Twenty ml. of 2% HPO_3-5% H_2SO_4 was then added, the flask was filled to the 100-ml. mark with eight per cent acetic acid, and after thorough mixing a five or 10-ml. aliquot was titrated with freshly standardized dye solution. The residual solid material from the boiling was extracted as described and a 25-ml. aliquot of the extract from the solids was used in the titration.

The 50-gm. samples were put on in cold distilled water, brought up to boiling in three minutes, and boiled 15 minutes, with two rates of boiling being used—a slow rate (barely bubbling) and a rapid rate (vigorous escape of steam). The vessel was removed from the flame and allowed to cool to room temperature. The liquids were separated, without washing the solids and adding washings to liquid as described above, the solids were extracted, and both portions were reduced with hydrogen sulphide as was done in the analysis before boiling.

DISCUSSION OF RESULTS

The 87 samples of raw turnip greens used for the 2.5-gm. tests averaged 200.5 mg. of ascorbic acid, with a maximum of 422 and a minimum of 67 mg. per 100 gm. The 54 samples for the 50-gm. samples averaged 190 before boiling with a maximum of 302 and a minimum of 125. The ascorbic acid in solution averaged 88, and in the residue 53 mg. per 100 gm. of the greens. When 2.5 gm. to 50 ml. of water were used, practically all the reduced ascorbic acid was found in the cooking liquor and 7.5 mg. per 100 gm. in the solids. When 50 gm. were boiled with 75 to 200 ml. of water, from one half to one third remained in the solids. The other data were

TABLE 1

Relation of Covering, Material of Dish, and Presence of Sodium Carbonate to Percentage of Reduced Vitamin C Changed

Cooking vessel	Reduced ascorbic acid lost or gained in percentage of reduced ascorbic acid at beginning		
	Covered	Uncovered	Loss owing to uncovering
Water only			
Aluminum cup	23.4	—27.0	27.0(50.4)
Enamel cup	—6.0	—28.8	22.8
Pyrex cup	—0.9	—27.0	26.1
In sodium carbonate, 1,000 p.p.m.			
Aluminum cup	—19.5	—66.5	47.0
Enamel cup	—9.8	—50.4	40.6
Pyrex cup	—9.8	—47.5	37.7

condensed and summarized (Tables 1, 2, and 3) in terms of percentage of the vitamin C which was gained or lost in the boiling.

From the data it is apparent that the greatest losses of reduced ascorbic acid in cooking the greens came from leaving the vessel uncovered when 2.5 gm. were used to 50 ml. of water (Table 1). On the other hand, when 50 gm. of greens were used in most of the tests (Table 3), losses from the covered vessels were greater than from the uncovered ones. The latter test more closely approaches kitchen conditions than the first tests.

There is little difference in the loss of reduced vitamin C from the 2.5-gm. portions when the cooking vessels are left uncovered, but with the 50-gm. portions, losses were lower on rapid boiling in uncovered pyrex, stainless steel, or enamel vessels than in the uncovered aluminum vessel. When sodium carbonate was present, the loss with the aluminum cup was greater than with the enamel or pyrex cups.

When 2.5 gm. were used and the vessels were covered, there was a gain of 23.4 per cent of reduced ascorbic acid in the aluminum cup when the distilled water was used, and losses of .9 to 6 per cent with the enamel or pyrex vessels. On the other hand, when 1,000 parts per million of sodium carbonate was present, the loss in the aluminum cup was twice that in the others, being 19.5 per cent compared with 9.8 per cent. When 50 gm. of greens were used (Table 3) the loss of total vitamin C was greater with the aluminum vessel than with the others.

Other workers have reported increases in titratable vitamin C during cooking in water, as was observed above with the aluminum cup, and have ascribed these increases to reduction of the dehydroascorbic acid or to hydrolysis of esterified ascorbic acid; while others have thought no real increase takes place but that the difference is due to checking of loss owing to action of the enzymes in the cooked portion, while the enzymes decreased the quantity in the uncooked portion. As pointed out by Borsook, Davenport, Jeffreys, and Warner (1936) ascorbic acid may be reversibly oxidized to dehydroascorbic acid, which in turn may be converted irreversibly into decomposition products. Although it has been claimed by Mack (1936) that ascorbic acid exists in fresh vegetables only in the reduced state, the increase in titratable vitamin C obtained on boiling turnip greens in water in a covered aluminum vessel may be readily explained if some of the vitamin C present in the raw greens is present as dehydroascorbic acid. Boiling in water in aluminum in the absence of oxygen reduces any dehydroascorbic acid to titratable ascorbic acid. Only the aluminum, not the pyrex or the enamel, brings about this reduction. Sodium carbonate inhibits this reduction by aluminum and tends to destroy the ascorbic acid present, probably hydrolyzing the lactone ring and initiating more deep-seated changes. Although no enzymic oxidation of the reduced ascorbic acid occurs during the heating—Kertesz, Dearborn, and Mack (1936) have shown that any ascorbic acid oxidase present in the leaves would be completely inactivated by one minute of boiling—it is obvious that boiling with free access to atmospheric oxygen oxidizes or decomposes large percentages of the titratable ascorbic acid originally present. This effect is much greater when combined with the sodium carbonate effect.

Since the large increase in reduced ascorbic acid with the 2.5-gm. samples occurs in the aluminum cup and not in the pyrex or enamel vessel, the most probable explanation seems to be the reduction of dehydroascorbic acid. The results with the larger samples are different, a loss occurring with the aluminum vessel.

The effect of sodium bicarbonate or sodium carbonate added to the water is shown (Table 2). When the greens were boiled in a covered aluminum dish (Table 3), the presence of 100, 200, or 500 parts per million of sodium bicarbonate had practically no effect upon the reduced ascorbic acid content when compared with the original greens, but when these results are compared with those for distilled water, it is found that these additions inhibit the increase in reduced vitamin C which occurs in distilled water. Additions of 1,000 parts per million of sodium bicarbonate or sodium acetate in acid or neutral solution cause a decrease of 6.6 to 8.4 per cent of reduced ascorbic acid compared with the original material, and about 31.8 to 30 per cent decrease when the increase with distilled water is considered.

TABLE 2

Effect of Added Salts on Loss of Reduced Vitamin C in Boiling in Covered Aluminum Cup

Solution	Loss or gain of original reduced ascorbic acid
	pct.
Distilled water	23.4
Sodium bicarbonate	
100 p.p.m.	4.7
200 p.p.m.	1.2
500 p.p.m.	—2.2
1,000 p.p.m.	—8.4
Sodium acetate	
From 1,000 p.p.m. sodium bicarbonate, neutral	—7.5
From 1,000 p.p.m. sodium bicarbonate, acid	—6.6

The data (Table 2) show that reduced ascorbic acid decreased about 20 per cent when in the presence of 1,000 parts per million of sodium carbonate and a covered aluminum cup was used, and about 10 per cent when covered enamel or pyrex cups were used. If uncovered vessels were used, the loss in the presence of 1,000 parts per million of sodium carbonate was about 66.5 per cent in the aluminum cup and 50 per cent in the enamel or pyrex vessel, compared with about 27 per cent in the distilled water.

The data obtained using 50-gm. samples and commonly used proportions of water are summarized (Table 3), each figure being the average of three or four tests. There are average losses of from 25.3 to 26.9 per cent of total vitamin C during boiling in aluminum vessels, from 15.5 to 32.6 per cent in enamel, from 17.1 to 36.5 per cent in pyrex, and 18.3 to 36.2 per cent in stainless steel. The total

losses in vitamin C in aluminum are independent of the rate of boiling and of the covering or uncovering of the vessel. The losses owing to slow boiling in the other kinds of dishes can be materially decreased by speeding up the boiling rate and again by uncovering the vessel. The losses owing to rapid boiling in uncovered enamel are 10.6 per cent less than the corresponding losses in aluminum.

These results indicate that covering the enamel pan appreciably promotes the irreversible decomposition of vitamin C during boiling

TABLE 3

Effect of Kind of Vessel, Covering, and Rate of Boiling on Ascorbic Acid

Cooking vessel	Total ascorbic acid lost in percentage of total ascorbic acid at beginnng		
	Rapid boiling	Slow boiling	Effect of increasing rate of boiling
Aluminum			
Uncovered	26.1	25.5	0.6
Covered	26.9	25.3	1.6
Loss owing to covering	0.8	—0.2	.....
Enamel			
Uncovered	15.5	26.6	11.1
Covered	26.7	32.6	5.9
Loss owing to covering	11.2	6.0	.....
Pyrex			
Uncovered	17.1	23.8	6.7
Covered	24.2	36.5	12.3
Loss owing to covering	7.1	12.7	.....
Stainless steel			
Uncovered	18.3	30.7	12.4
Covered	26.2	36.2	10.2
Loss owing to covering	7.9	5.5	.....

of turnip greens, probably because of the higher temperature of boiling when covered. The freer access to the oxygen of the air made possible by slowing down the boiling rate has the same effect. Covering with a light-weight aluminum cover, which is itself a reducing material, produces no increase in irreversible decomposition of vitamin C in the aluminum vessel. Neither does the increase in the oxygen concentration which attends the slowing down of the boiling rate promote the irreversible decomposition of vitamin C in the aluminum vessel.

For the purpose of preserving most of the total vitamin C in a biologically active form, the best method of cooking the turnip greens in pure water in average-sized amounts is by boiling rapidly in an

uncovered enamel vessel. If the heating must be done over a slow flame, however, a covered aluminum pan is better than one of enamel.

SUMMARY

Turnip greens (141 samples) contained from 67 to 422 mg. ascorbic acid per 100 grams, and when 50 gm. were cooked in 75 to 200 c.c. of water, on an average 88 mg. were in solution and 53 mg. were in the greens per 100 grams of turnip greens.

Losses of total vitamin C under conditions similar to practical cooking ranged from 15.5 to 26.7 per cent on rapid cooking, and from 23.8 to 36.5 per cent on slow cooking.

There were lower losses in uncovered enamel, pyrex, or stainless steel vessels when the greens were cooked rapidly, than when they were cooked slowly. The losses were the same (25.3 to 26.9 per cent) when aluminum was used, covered or uncovered.

An increase in titratable vitamin C occurred when 2.5 gm. of greens were cooked in 50 ml. of water in covered aluminum vessels, but this increase was probably due to reduction of reversibly oxidized ascorbic acid, which is biologically active.

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BEHAVIOR OF MICROÖRGANISMS AT SUBFREEZING TEMPERATURES

I. FREEZING REDISTRIBUTION STUDIES[1, 2]

VERNON H. McFARLANE
Food Research Division, Bureau of Agricultural Chemistry and Engineering, U. S. Department of Agriculture, Washington, D. C.

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Investigations on the effect of freezing temperatures on microbial life have hitherto been largely qualitative in that they have dealt mainly with the question of whether or not all microörganisms were destroyed. Interpretations of survival or destruction have usually been based upon examination of well-mixed portions of the *whole* mass before freezing and after thawing.

The conception held in the present work is that within the frozen mass, whether it be water, syrup, brine, juice, syrup-packed fruit, or brine-packed vegetable, conditions are far from homogeneous. Such factors as manner and rate of crystallization, density and viscosity of the medium, gravity, mechanical obstructions, etc., all tend to influence redistribution of suspended and dissolved substances in a fluid medium during the freezing process. The assumption seems warranted that if the conditions are not uniform throughout the frozen mass, there may be within the mass certain areas relatively favorable for microbial survival or microbial destruction. These conditions would arise most freely when fluid media are frozen solidly in undisturbed containers and would prevail under such conditions as are common in frozen-pack practice.

It is recorded in the literature that clear ice contains fewer microbial forms and less soluble mineral matter than ice containing air bubbles. Studies responsible for such data have for the most part been on river, lake, or pond ice. During the freezing of such bodies of water some self-purification takes place when the crystallizing surface water forces impurities into the fluid water beneath. Sectional studies on a given container of frozen fluid material stored at a freezing temperature are, however, lacking. The relationship between microbial distribution and destruction and the distribution

[1] Food Research Division Contribution No. 439.

[2] This paper was condensed from a portion of a thesis submitted in partial fulfillment of requirements for the degree of Doctor of Philosophy, University of Washington, 1938. Many tables, plates, and details of technic were omitted. Discussions were shortened or eliminated. The investigations were made in the Frozen Pack Laboratory of the Bureau of Agricultural Chemistry and Engineering in Seattle, Washington.

of such soluble substances as salts, sugars, and acids has not been conclusively established.

REVIEW OF THE LITERATURE

Pengra (1882) was interested in freezing as a mechanism of purification. Solutions containing known quantities per unit volume of urea, of grape sugar, and of arsenic were partially or solidly frozen; and samples from frozen and unfrozen portions or from different layers of the ice masses were analyzed. Later, Pengra (1884), portions of suspensions of bacteria, of *Coleps hirtus*, of *Paramaecium aurelia*, and of *Glaucoma scintillans* were frozen and the extent and nature of freezing purification determined by microscopic methods. Prudden (1887) found that snow ice and very "bubbly" ice contained a much greater number of bacteria than adjacent transparent ice from the same block. Christomanos (1898) froze at —14°C. (6.8°F.) a liter of drinking water containing 71 bacilli per c.c. Eight hundred and twenty grams of clear ice formed; 170 grams of core ice and eight to 10 grams of water remained unfrozen. There were only eight to 15 bacilli per c.c. in the clear ice, while 450 were found in the core ice. Clark (1901) like Pengra, was largely interested in the freezing purification of water from the public-health standpoint. Most of his bacterial and chemical analyses were run on ice and water samples from tanks, ponds, and rivers. He froze suspensions of bacteria in relatively small containers and demonstrated freezing purification or redistribution.

When cider or milk is frozen there is a concentration or redistribution of the solids. Frozen barrelled cider has been excellently described by Gore (1914) and frozen milk masses by Reid (1927) and Baldwin and Combs (1932). Woodroof (1931) examined samples of slow-frozen grape juice taken from different positions in a pound package. He found that such samples showed a variance from 16 per cent sugar on the side to 62 per cent at the top and 57.7 per cent at the bottom. Hess (1934) froze *Pseudomonas fluorescens* slowly in distilled water [—16°C.(3.2°F.) in air] and examined separately the frozen liquid portions in successive tubes by means of plate counts. His conclusion, like that of others, was that during the freezing process the bacteria are extruded from the solid into the liquid portion of the medium. Young (1934) observed the central concentration of cresoap emulsions frozen in test tubes at —3 to —30°C.(26.6 to —22°F.). He also observed that when a .25-per cent amorphous carbon suspension was frozen in test tubes carbon particles and gas bubbles were concentrated in long narrow pockets in the centers of the ice columns.

EXPERIMENTAL METHODS

Uniform suspensions and solutions were frozen in fibreboard cylinders in still air. An effort was made to determine the actual distribution of the suspended and soluble substances in the frozen masses. Therefore, macroscopic, photographic, and laboratory examinations were made of central slabs, and of numerous small sections from these slabs representing all the different areas. It was hoped that the information so derived might throw some light upon possible microbial distribution and also upon environmental variances within a given frozen mass.

Waxed cylindrical fibreboard containers, "malt quarts," 3⅜ x 6⅛ inches (8.57 x 15.56 cm.) of over 800-c.c. capacity, were selected for the experimental work because of (a) their convenient size, (b) their actual use commercially or similarity to other containers used in the commercial freezing of fruits, vegetables, and other products, and (c) the fact that frozen contents could be partially sectioned without first removing the fibreboard cylinders.

Fluids were dispensed in 750-c.c. quantities and frozen and stored at —20°C.(—4°F.); a few samples also at —10°C.(14°F.). These temperatures were readily and permanently available. The quantity of fluid dispensed left a headspace of 20-30 mm. for freezing expansion. In the cold-storage rooms the filled and closed containers were placed on floor racks to enable uniform contact with cold air on all sides. No effort was made to circulate the cold air but some air movement in the rooms was undoubtedly brought about by the air currents set up on entrance or the convection currents caused by the higher temperatures of the uncooled packs.

All sectioning of the frozen masses was done in the —20°C. room. A small band saw equipped with an adjustable carriage enabled the cutting of a central vertical ice slab and smaller pieces (Figs. 1 and 2).

PHOTOGRAPHIC STUDIES

The substances frozen, sectioned, and photographed were tap water, one- and two-per cent sodium chloride solutions, and one-, five-, and 10-per cent sucrose solutions, with and without india ink. Commercial grades of salt (sodium chloride) and cane sugar (sucrose) were dissolved in cold tap water in the quantities necessary to make the percentage solutions desired. Higgins' waterproof black ink was suspended in tap water and in the solutions in the proportion of one c.c. per 1,000 c.c. of suspending medium. The various mixtures were thoroughly agitated, dispensed, and immediately taken to cold storage while still at a temperature of 12 to 14°C.(53.6 to 57.2°F.).

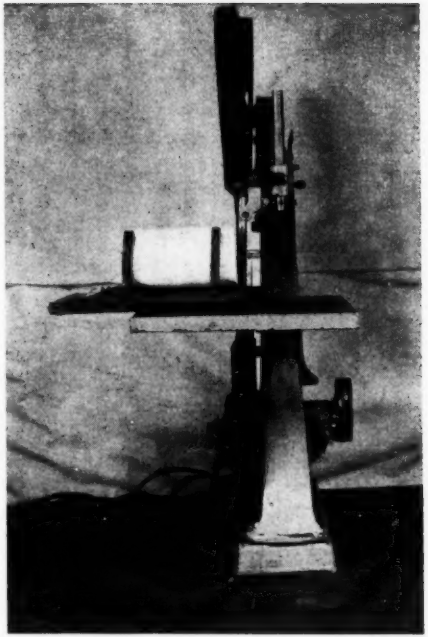

FIG. 1. Band saw carriage for cutting the central vertical slab from the ice mass.

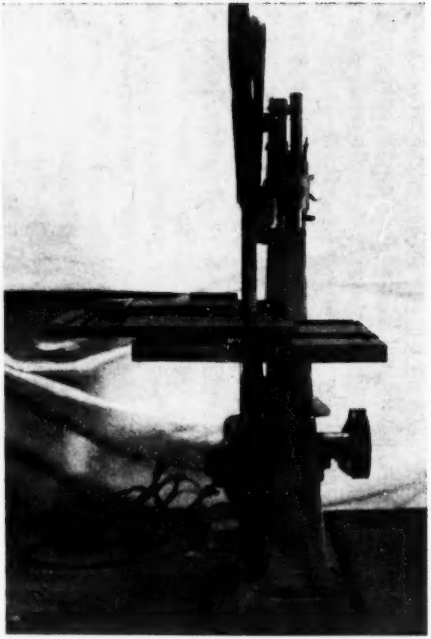

FIG. 2. Band saw carriage for sectioning the central vertical slab. (Slab in place for sectioning.)

After the above-described solutions and suspensions had been in freezing storage for one week, central vertical slabs one centimeter thick were cut from them and photographed. Four of the photographs are shown (Figs. 3, 4, 5, and 6).

In the frozen water (Fig. 3) the core of opaque ice is a centrally located, cylindrical core structure having a diameter approximately one-third that of the whole ice mass. It does not extend through the entire ice mass but starts near the bottom and extends vertically to within about half an inch of the top. Expansion of the water last frozen increases the diameter of the upper part of the core. In its upper extremity the core becomes more typically cone-shaped. The tip slightly expands and appears to rest against a horizontally striated layer of ice about one-half inch in thickness. Radiating rays, which are long ice-enclosed air bubbles, extend like spines in all directions from the core. They extend through the peripheral ice only at the base, otherwise only about half way. Upper "spines" are more or less perpendicular to the surface of the opaque ice. Lower ones bend slightly downward to directly downward at the base. India ink particles are for the most part concentrated in and on the opaque ice (Fig. 4). A considerable number are trapped in the surface layer of ice and help give rise in the cut section to horizontal striations. There is a halo or zone of clear ice around the upper portion of opaque ice which is particularly noticeable in photographs of water frozen at —10°C.(14°F.). It is between the opaque and radiating ray areas. It surrounds or else is a part of the area last frozen and it intensifies the swollen appearance of the upper part of the core.

In a frozen sodium chloride or sucrose solution a central core of opaque ice possessing regularity of shape is not so much in evidence. Central areas possess a greater opaqueness. When india ink is added and the solution frozen, the cone-shaped nature of the core is emphasized (Figs. 5 and 6). Radiating rays are not visible but a zoning effect or layering is noticeable. The concentric striations responsible for this effect are made more prominent by entrapped india ink particles. The concentration of the solute in the frozen mass free of india ink is not so obvious.

The macroscopic and photographic studies made demonstrated the influence of different concentrations of solute on the freezing redistribution of india ink particles. India ink appeared to be concentrated more in a frozen one-per cent sodium chloride suspension than in a two-per cent; more in a frozen one-per cent sucrose suspension than in a five-per cent and more in a five-per cent than in a 10-per cent. Effect of temperature could also be noted, although

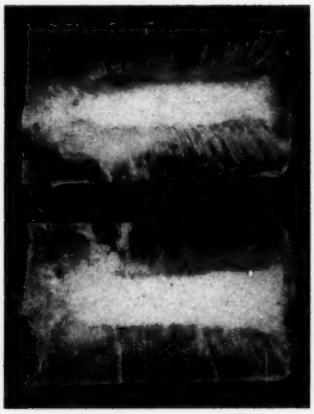

FIG. 3. Tap water frozen and stored at —20°C. (—4°F.); at —10°C.(14°F.).

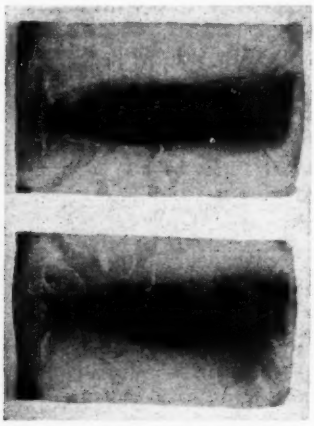

FIG. 4. India ink suspended in tap water, frozen, and stored at —20°C.(—4°F.); at —10°C. (14°F.).

FIG. 5. India ink suspended in a two-per cent sodium chloride solution, frozen, and stored at —20°C.(—4°F.); at —10°C. (14°F.).

FIG. 6. India ink suspended in 10-per cent sucrose solution, frozen, and stored at —20°C. (—4°F.); at —10°C.(14°F.).

the nature of the soluble and of the suspended material did not always permit an accurate judgment of the areas of greatest concentration. The cone-shaped cores were not always strictly vertical or identical in position. Saw marks are conspicuous on the —20°C. (—4°F.) ice slab (Fig. 3) and on both slabs (Fig. 5).

SECTIONAL STUDIES

Two-per cent sodium chloride solution, 10-per cent sucrose solution, dilute hydrochloric acid solution (pH 3.22), and acidified

FIG. 7. Sectioned ice slab.

10-per cent sucrose solution (pH 3.22, adjusted with HCl) were dispensed and frozen in "malt quart" containers at —20°C. A central vertical slab 1.5 cm. thick was cut from each container. Each slab was first cut into five longitudinal strips or zones—two peripheral, two intermediate, and one core. The longitudinally cut strips were then turned on the carriage and nine cuts made transversely so that 50 small blocks or sections resulted (Fig. 7). The positions of the sections in the ice mass were indicated by numbers (Fig. 8). Dimensions varied slightly, even for the more regular blocks, from 1.5 x 1.5 x 1.2 cm. Corresponding peripheral sections were placed in the same numbered vial. Corresponding intermediate sections were similarly treated. Core sections, being unpaired, were each put in

individual numbered vials. In all, 30 samples, representative of every part of the ice mass, were prepared for examination from the frozen contents of each "malt quart." The stoppered vials were removed from the cold-storage room, the contents were thawed and examined. Percentage of salt or sugar in a thawed section was determined with a refractometer and the pH was determined with a glass electrode pH meter; findings are reported (Tables 1, 2, 3,

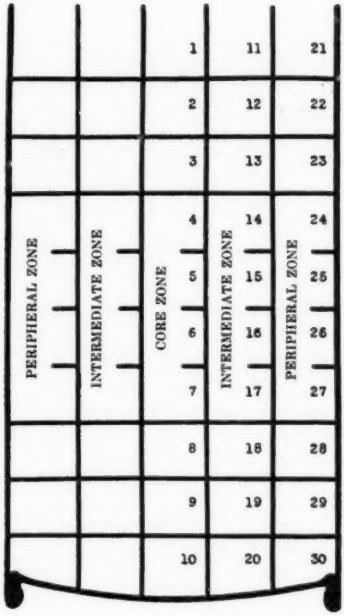

FIG. 8. Diagram of plan for sectioning.

and 4). Data are arranged in each table in the same order as the positions of the sections in the frozen mass beginning at the top (Fig. 8). Each value was determined by averaging the readings made on corresponding sections from several "malt quart" samples, 10 samples each for Tables 1 and 2, seven for Table 3, and six for Table 4.

The tabulated data need little discussion. Top sections, 1, 11, and 21, in most instances showed higher concentrations of solute than those sections immediately below. Three factors responsible for this were (a) freezing redistribution, (b) freezing expansion, and (c) manner of sectioning.

DISTRIBUTION OF YEAST CELLS IN FLUID MEDIA WHEN FROZEN

It was desirable to demonstrate the possibility that microbial forms might be distributed in fluid media when frozen in the same manner as india ink particles. With this in mind, yeast-cell suspen-

TABLE 1

Sectional Distribution of Sodium Chloride When a Two-Per Cent Solution Is Frozen at —20°C.(—4°F.)

Core zone	Intermediate zone	Peripheral zone
pct.	*pct.*	*pct.*
1.6	1.7	1.8
1.1	0.7	0.3
2.6	1.5	0.3
4.7	2.4	0.4
5.8	2.7	0.4
6.3	2.9	0.4
6.4	3.1	0.5
6.5	3.3	0.6
6.0	3.5	0.7
4.0	3.1	1.2

TABLE 2

Sectional Distribution of Sucrose When a 10-Per Cent Solution Is Frozen at —20°(—4°F.)

Core zone	Intermediate zone	Peripheral zone
pct.	*pct.*	*pct.*
11.9	13.9	19.7
6.7	6.0	3.7
11.8	10.9	4.2
21.0	16.7	4.9
28.0	19.5	5.5
32.6	21.0	5.9
34.8	21.7	6.3
35.5	22.5	6.8
33.9	22.3	7.0
24.4	20.7	9.5

TABLE 3

Sectional Distribution of H-Ions When a Dilution of HCl in Tap Water Is Frozen at —20°C.(—4°F.)

Core zone	Intermediate zone	Peripheral zone
pH	*pH*	*pH*
5.21	4.53	4.27
5.10	5.29	6.05
3.81	4.39	6.02
3.20	4.01	5.62
3.09	3.90	5.49
2.91	3.85	5.78
2.66	3.63	5.26
2.49	3.49	5.55
2.53	3.49	5.86
3.28	3.70	6.11

TABLE 4

Sectional Distribution of H-Ions When a Dilution of HCl in 10-Per Cent Sucrose Solution Is Frozen at —20°C.(—4°F.)

Core zone	Intermediate zone	Peripheral zone
pH	*pH*	*pH*
3.29	3.12	3.04
3.73	3.71	4.09
3.31	3.35	3.96
2.95	3.13	3.83
2.74	3.00	3.90
2.63	2.92	3.95
2.57	2.93	3.96
2.62	2.88	3.78
2.68	2.81	3.77
2.97	2.84	3.66

sions were frozen and the frozen masses examined for cell distribution. The Saccharomyces species isolated by Berry (1934) from cider stored at —2.2°C.(28.2°F.) was used.

Washed yeast cells, about a half-million per cubic centimeter, were suspended in cold tap water, in two-per cent sodium chloride

solution, and in 10-per cent sucrose solution. All the suspensions were of approximate neutrality, pH 6.5-7.0. These suspensions were dispensed in "malt quarts" and frozen and stored in still air at —20°C.(—4°F.). Temperature of the suspensions at the time of transference to the cold room was 13°C.(55.4°F.). Frozen masses were sectioned as in the preceding study. The stoppered sample vials were transferred to the laboratory where the contents were thawed and thoroughly mixed. Haemocytometer counts were made on each section as rapidly as possible after thawing, and the number of cells per cubic centimeter was recorded. Average section counts for the different frozen suspensions are given (Tables 5, 6, and 7).

TABLE 5

Sectional Distribution of Yeast Cells Frozen in Tap Water at —20°C. (—4°F.)

Core zone	Intermediate zone	Peripheral zone
520,000	323,000	131,000
557,000	120,000	42,000
1,753,000	880,000	61,000
1,542,000	1,423,000	35,000
957,000	1,495,000	30,000
726,000	1,207,000	45,000
550,000	1,332,000	66,000
529,000	1,042,000	152,000
622,000	957,000	348,000
1,017,000	915,000	370,000

TABLE 6

Sectional Distribution of Yeast Cells Frozen in Two-Per Cent Sodium Chloride Solution at —20°C.(—4°F.)

Core zone	Intermediate zone	Peripheral zone
461,000	347,000	204,000
397,000	495,000	413,000
337,000	339,000	403,000
301,000	400,000	459,000
309,000	404,000	481,000
264,000	399,000	484,000
283,000	398,000	478,000
272,000	412,000	469,000
388,000	433,000	606,000
768,000	772,000	755,000

Redistribution of yeast cells in frozen tap water (original count 472,000 per c.c.) varied in different sections from 30,000 to 1,753,000 cells per c.c. of thawed ice. Greatest concentration of cells was in the core zone, Sections 3 and 4, and in the intermediate zone, Sections 14, 15, 16, 17, and 18. Counts were obtained on only four samples. Clumping interfered with accurate counting. The clumps became firmer with increased storage time; therefore, the values given (Table 5) are to be taken more as an indication of distribution trend than as an exact measure of cell distribution; compare Figs. 3 and 4 (—20°C. slabs) and Table 5.

Redistribution of yeast cells in two-per cent sodium chloride solution (original count, 557,000 cells per c.c.) varied in different sections from 204,000 to 772,000 cells per c.c. of fluid. Greatest concentrations of cells occurred in Sections 1, 10, 12, 20, 24, 25, 26, 27, 28, 29, and 30. The bottom sections, 10, 20, and 30, were highest in yeast-cell content. Haemocytometer counts were made on nine samples.

Clumping interfered only slightly. Yeast cells were not redistributed in the frozen mass in the same manner as the salt. Areas of greatest concentration of india ink particles, as shown in the photograph, were not necessarily areas of greatest yeast-cell concentration; compare Fig. 5 (—20°C. slab) and Tables 1 and 6.

Redistribution of yeast cells frozen in 10-per cent sucrose solution (original count, 595,000 cells per c.c.) varied in different sections from 444,000 to 1,062,000 cells per c.c. thawed ice. Greatest concentration of cells was in the lower core zone, Sections 6, 7, 8, 9, and 10. High concentrations of cells also occurred in Sections 1, 5, 11, 15, 16, 17, 18, 19, 20, and 30. Haemocytometer counts were made on nine samples. Clumping did not interfere with accurate counting. Freez-

TABLE 7

Sectional Distribution of Yeast Cells Frozen in 10-Per Cent Sucrose Solution at —20°C.(—4°F.)

Core zone	Intermediate zone	Peripheral zone
707,000	692,000	544,000
514,000	519,000	444,000
493,000	534,000	490,000
657,000	622,000	474,000
813,000	720,000	504,000
926,000	722,000	504,000
983,000	736,000	516,000
1,024,000	791,000	502,000
1,011,000	766,000	534,000
1,062,000	945,000	697,000

ing redistribution of yeast cells was similar to the freezing redistribution of the sucrose except that while the bottom core section rarely contained the greatest concentration of sucrose, it almost always contained the highest concentration of yeast cells. Freezing redistribution of yeast cells compared favorably with that of india ink particles; compare Fig. 6 (—20°C. slab) and Tables 2 and 7.

DISTRIBUTION OF VIABLE YEAST CELLS IN FLUID MEDIA WHEN FROZEN FOR DIFFERENT TIME INTERVALS

This study is distinguished from the previous two in that it is concerned with (a) the distribution of viable yeast cells in a given frozen mass, i.e., those cells capable of colony formation on wort agar plates; and (b) the effect of storage time on viability of the yeast cells in different sections of the same frozen mass.

Suspensions of approximately a half-million yeast cells per cubic centimeter were made in tap water, in two-per cent sodium chloride

solution, in 10-per cent sucrose solution, in dilute hydrochloric acid solution (pH 3.22) and in acidified 10-per cent sucrose solution (pH 3.22, adjusted with HCl). These were dispensed in 750-c.c. quantities in "malt quart" containers and frozen in still air at —20°C. At regular intervals of time, usually every two weeks for the first three months, frozen samples were sectioned. Sections were placed in sterile stoppered vials, taken to the laboratory and thawed in the refrigerator at 5°C.(41°F.). Suitable dilutions were then made for each thawed section. Each dilution was plated in duplicate on wort agar, pH 4.8. Plates were incubated for five days at 22°C.(71.6°F.). Only gross comparisons were made between total and viable cell content of a given section. The term "progressive," as used in the following paragraphs to modify decrease (in viable cells), refers to the tendency for viable microbial forms in frozen media to decrease in number with increase in the length of the storage period, and is not intended to describe the rate of decrease.

Distribution of Viable Yeast Cells in Frozen Tap Water: During the 46-week period 11 samples were sectioned and plated. At the end of six weeks section counts ranged from 8,600 viable cells per c.c. of thawed section to 672,000. At the end of 46 weeks the counts ranged from 1,800 to 417,500. Survival was greatest in the lower seven sections of the intermediate zone and in Sections 3, 4, and 10 of the core zone. There was a progressive decrease of viable cells in each part of the ice mass with the passage of time. The gross difference in sectional distribution between the six-week and the 46-week samples was largely one of degree, the distribution pattern being approximately the same.

Distribution of Viable Yeast Cells in Frozen Two-Per Cent Sodium Chloride Solution: At the end of six weeks sections counts ranged from 1,900 to 132,000; at the end of 46 weeks, 500 to 15,800. The greatest number of viable cells at the end of the period occurred in the lower nine peripheral sections and in Sections 10 and 20 of the core and intermediate zones. The general plan of viable cell distribution closely paralleled that of total cell distribution. All the sections having a high viable cell count, with the exception of 10 and 20, were low in sodium chloride.

Distribution of Viable Yeast Cells in Frozen 10-Per Cent Sucrose Solution: At the end of six weeks section counts ranged from 175,000 to 675,000 and at the end of 46 weeks from 10,750 to 155,000. At 46 weeks the yeast-cell survival was greatest in the lower five sections of the core zone and in the bottom sections of the intermediate and peripheral zones. These sections were high in sucrose.

Distribution of Viable Yeast Cells in Frozen Dilute Hydrochloric Acid Solution: During an 18-week storage period 12 samples were sectioned and plated. At the end of one week section counts ranged from 7,200 to 1,800,000; at the end of 18 weeks, from 14 to 289,000. A progressive decrease in the number of viable cells per section was noted. Yeast-cell survival was greatest at the end of 18 weeks in the lower eight sections of the intermediate zone and in Sections 3 and 10 of the core zone. Destruction of cells was most complete and most rapid in Sections 7, 8, and 9, which were high in hydrogen-ion concentration.

Distribution of Viable Yeast Cells in Frozen Acidified Sucrose Solution: Section counts ranged from 18,000 to 98,000 at the end of one week and from five to 1,090 at the end of 18 weeks. Cells tended to survive in greater numbers in sections of the peripheral zone (except in Section 21) and in the lower sections of the core and intermediate zones. Greatest destruction of viable cells occurred during the first month, and while destruction was rapid in all sections, it was especially so in sections of low pH in spite of their high sucrose concentration. Rate of destruction was much slower during the remainder of the storage period. There was a relatively high hydrogen-ion concentration in all sections (Table 4). The viable cell-distribution pattern differed from the total cell-distribution pattern characteristic for the medium.

TABLE 8

Viable Yeast Cells in Media Frozen and Stored at —20°C.(—4°F.) for Different Time Intervals

Suspending medium	Plate count before freezing	Plate count after freezing			
		2 weeks	8 weeks	18 weeks	46 weeks
Tap water, pH 6.5-7.0	435,000	197,000	178,000	.....	119,000 [1]
2% sodium chloride sol., pH 6.5-7.0	481,000	185,000	107,000	.....	15,800 [1]
10% sucrose sol., pH 6.5-7.0	519,000	343,000	253,000	.....	138,800 [1]
Tap water, pH 3.22	453,000	207,000	107,000	69,700 [1]	.....
10% sucrose sol., pH 3.22	458,000	8,730	1,300	311 [1]	.....

[1]Average of plate counts made on three samples.

Viable Yeast Cells in Tap Water, Two-Per Cent Sodium Chloride Solution, 10-Per Cent Sucrose Solution, Dilute Hydrochloric Acid Solution, and Acidified Sucrose Solution Frozen and Stored at —20°C.: When entire (unsectioned) samples of the frozen suspensions were examined for the presence of viable cells, it was found that yeast-cell survival was greatest after a long period of freezing storage in two media of approximate neutrality, water and 10-per cent sucrose solution. Yeast-cell survival was least in 10-per cent

sucrose solution, pH 3.22. The presence of hydrogen ions had a definite effect upon the rate of yeast-cell destruction (Table 8).

SUMMARY

When 750-c.c. quantities of water in "malt quarts" were frozen in still air at —10 or —20°C.(14 or —4°F.) central, vertical, cone-shaped cores of opaque ice and upper horizontal layers were formed. India ink particles suspended in the water were concentrated, by freezing, in the core ice and in and on the horizontal layer. The presence of a solute in the suspending medium (one- or two-per cent sodium chloride or one-, five-, or 10-per cent sucrose) retarded the degree of concentration and modified the distribution pattern of the india ink particles in the frozen mass.

Sodium chloride (two per cent), sucrose (10 per cent), and dilute hydrochloric acid (pH 3.22), each frozen in 750-c.c. quantities of solution, were similarly concentrated in cone-shaped cores and horizontal layers. The presence of sucrose (10 per cent) in a dilute hydrochloric acid medium (pH 3.22) interfered with the degree of hydrogen-ion redistribution.

Yeast cells suspended in tap water, in two-per cent sodium chloride solution and in 10-per cent sucrose solution, dispensed in 750-c.c. quantities in "malt quarts" and frozen in still air at —20°C. were concentrated in the frozen mass. The yeast-cell distribution pattern differed for each suspending medium. When the medium was of approximate neutrality there was, with increasing length of storage, a progressive decrease of viable cells in all parts of the ice mass. The distribution pattern for viable cells on a given date closely resembled the distribution pattern characteristic for the total cells. However, the presence of hydrogen ions in a medium hastened the destruction of yeast cells and sometimes markedly altered the distribution pattern of these cells in the frozen mass.

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BEHAVIOR OF MICROÖRGANISMS AT SUBFREEZING TEMPERATURES

II. DISTRIBUTION AND SURVIVAL OF MICROÖRGANISMS IN FROZEN CIDER, FROZEN SYRUP-PACKED RASPBERRIES, AND FROZEN BRINE-PACKED PEAS [1, 2]

VERNON H. McFARLANE
Food Research Division, Bureau of Agricultural Chemistry and Engineering, U. S. Department of Agriculture, Washington, D. C.

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In a previous publication by McFarlane (1940) redistribution of microbial forms was demonstrated when uniform suspensions in water and in aqueous solutions were frozen. A redistribution of soluble solids was also demonstrated. It was shown that microbial forms retained their viability for long periods of time in those sections of a frozen mass which possessed a high concentration of a favorable substance such as sucrose. On the other hand, it was also shown that certain concentrations of hydrogen ions or total acids were deleterious even in the presence of sucrose. The relationships between redistributed microbial forms and the varied environmental conditions in a given frozen mass were briefly considered for limited experimental conditions. Only unbuffered liquids were employed as suspending media in the experiments hitherto conducted. For this reason, studies were undertaken on the distribution and survival of microörganisms in packs of frozen cider, frozen syrup-packed raspberries, and frozen brine-packed peas. It was hoped that the findings might enable a better understanding of the microbiology of similar commercially frozen food products. The packs satisfied the need for buffered and for two-phased media. The effect of buffer substances on the freezing redistribution of hydrogen ions was not determined in the previous work. Neither were the microbial distribution and the environmental range in a frozen mass determined for a two-phase (liquid and solid) suspending medium. Evidence was not obtained which would indicate whether or not large quantities of insoluble substances might have any influence on the freezing and gravitational redistribution of microörganisms, soluble solids, hydrogen ions, etc., in the liquid phase of the medium.

[1] Food Research Division Contribution No. 440.

[2] This paper was condensed from a portion of a thesis submitted in partial fulfillment of requirements for the degree of Doctor of Philosophy, University of Washington, 1938. The investigations were made in the Frozen Pack Laboratory of the Bureau of Agricultural Chemistry and Engineering in Seattle, Washington.

Freezing and sectioning technics used in the following experiments were not essentially different from those previously reported by McFarlane (1940).

FROZEN CIDER

Fresh apple cider was thoroughly mixed and dispensed in 750-c.c. quantities in "malt quarts," waxed cylindrical fibreboard containers, 3⅜ x 6⅛ inches (8.57 x 15.56 cm.) in size. The containers were immediately taken to the cold-storage rooms, half to —10°C.(14°F.)

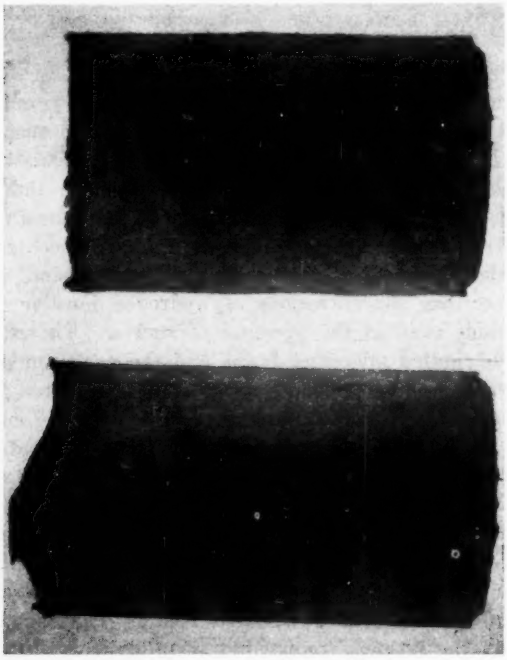

FIG. 1. Central vertical slabs cut from cider frozen and stored at —20°C. (—4°F.); at —10°C.(14°F.).

and half to —20°C.(—4°F.). They were set on floor racks so that the air could come in contact equally with all sides. Temperature of the cider at the time of storing was 14°C.(57.2°F.).

Wort agar plate counts made on well-mixed composite samples of cider just prior to freezing showed the natural microbial content, largely yeasts and molds, to be 803,000 per c.c. The refractometer reading for total soluble solids was 10.6 per cent and the glass electrode reading for hydrogen-ion concentration was pH 3.5. Total

acidity as measured by N/100 NaOH and phenolphthalein was 7.36 c.c. per one c.c. of sample.

Whole samples frozen and stored at the two temperatures were examined from time to time to determine the number of viable microbial cells surviving the various periods of storage. Other samples stored at —20°C. were sectioned and 30 typical sections of each were analyzed for viable microbial forms, percentage of soluble solids, total acid content, and hydrogen-ion concentration. Samples stored at —10°C. were too soft to section but were drained, and analyses of frozen and unfrozen portions were compared with the analysis of the whole (Tables 1, 2, 3, 4, 5, and 6). Data are arranged in the first four tables in the same order as the positions of the sections in the frozen mass beginning at the top. Recorded counts represent the number of cells per cubic centimeter for each thawed section.

The distribution of viable microbial forms in 750-c.c. quantities of cider dispensed in "malt quarts" and frozen and stored at —20°C. varied with the length of the storage period. At the end of the first week viable forms occurred in greatest numbers in the lower sections of the core and intermediate zones and in or on the surface sections. By the end of the 15th week surface and bottom sections contained the greatest number of viable microörganisms. Other sections were lower but fairly uniform in count, whether from the core, intermediate or peripheral zones. Between the 18th and the 31st week another change occurred in the sectional relationship of the viable microbial forms. Surface and bottom sections still remained relatively high in count, but peripheral sections ranked higher than corresponding core or intermediate sections, a reversal of the relationship originally displayed (compare Tables 1 and 2).

Soluble solids were redistributed in cider by freezing at —20°C. (Table 3). The heaviest concentrations were found in sections from the lower core and intermediate zones. Surface sections were relatively high in soluble solid content. The distribution pattern resembled that for 10 per cent sucrose, McFarlane (1940). Metacryotic liquid scraped from the surfaces of 12 samples gave refractometer readings ranging from 49.9 to 64.3 per cent, average 58.2. Very little redistribution of hydrogen ions was noted. Peripheral sections rarely exceeded core sections in pH by more than .2. Total acids were concentrated in the same manner as the soluble solids under the same experimental conditions (compare Tables 3 and 4). Findings with regard to freezing redistribution in fruit juices are in agreement with those of Gore (1914), Woodroof (1931), and Lutz, Caldwell, and Moon (1932).

TABLE 1

Sectional Distribution of Viable Microbial Forms[1] Frozen One Week in Cider at —20°C.(—4°F.)

Core zone	Intermediate zone	Peripheral zone
800,000	810,000	680,000
515,000	475,000	450,000
565,000	610,000	395,000
970,000	400,000	455,000
1,040,000	880,000	445,000
1,290,000	730,000	375,000
1,150,000	820,000	430,000
1,080,000	870,000	495,000
1,140,000	860,000	430,000
1,040,000	1,160,000	640,000

[1] Organisms per c.c.

TABLE 2

Sectional Distribution of Viable Microbial Forms[1] Frozen 31 Weeks in Cider at —20°C.(—4°F.)

Core zone	Intermediate zone	Peripheral zone
11,000	18,800	32,400
2,900	2,950	5,200
1,900	4,350	5,600
1,950	4,600	6,250
1,400	4,150	6,000
1,450	3,750	5,400
2,550	4,000	4,450
2,000	3,000	4,150
2,000	2,800	3,850
12,000	14,400	31,200

[1] Organisms per c.c.

TABLE 3

Sectional Distribution of Soluble Solids in Cider Frozen at —20°C. (—4°F.)[1]

Core zone	Intermediate zone	Peripheral zone
pct.	*pct.*	*pct.*
18.16	17.76	19.15
7.42	6.08	4.44
13.60	9.46	4.41
22.18	14.22	5.10
27.31	16.82	5.54
29.82	18.01	6.04
30.40	18.83	6.73
29.76	19.71	7.32
27.61	19.86	8.37
22.99	19.39	9.90

[1]Average readings made on thawed sections from 10 "malt quart" samples.

TABLE 4

Sectional Distribution of Total Acids in Cider Frozen at —20°C. (—4°F.)[1]

Core zone	Intermediate zone	Peripheral zone
12.1	11.8	10.9
5.3	4.2	3.1
9.6	6.5	3.0
15.5	9.6	3.5
19.0	11.5	3.8
20.8	12.6	4.2
21.0	13.2	4.8
20.8	13.9	5.2
19.3	13.7	5.7
15.3	13.4	6.6

[1]Average readings made on thawed sections from five "malt quart" samples; c.c. N/100 NaOH per c.c. cider.

TABLE 5

Viable Microbial Forms, Total Soluble Solids, and Total Acids in Cider Frozen and Stored 31 Weeks at —10°C.(14°F.)[1]

Sample	Plate count	Soluble solids (refractometer reading)	Total acids (c.c. N/100 NaOH per 1 c.c. sample)	Quantity of sample
		pct.		*c.c.*
Whole sample	46,100	10.86	7.59	684
Unfrozen portion	96,000	44.45	34.79	53
Frozen portion	41,500	7.44	5.15	629

[1]Average readings made on triplicate samples.

When samples held 31 weeks at —10°C.(14°F.) were drained, it was found that although the drainage was incomplete the viable microbial content of the unfrozen concentrate was much higher than that of the frozen portions; the soluble solid content was almost six times as great; the total acid content was more than six times as great (Table 5). Evaporation was responsible for some volume loss at —10°C.

Decrease of viable microbial forms in cider frozen and stored at —10 and —20°C. was progressive (Table 6). A comparison of temperatures with respect to the preservation of viable microbial forms showed that the greater preservation occurred in cider at the higher temperature. After 31 weeks 5.74 per cent were viable in cider at

TABLE 6

Microbial Viability in "Malt Quart" Samples of Cider Frozen and Stored at —10 and —20°C.(14 and —4°F.)

Sample No.	Storage temperature	Plate count before freezing	Plate count after freezing				
			1 week	2 weeks	6 weeks	15 weeks	31 weeks
	°C.						
47	—10	803,000	705,000	580,000	315,000	131,000	46,100
Per cent viable	.....	100.00	87.80	72.23	39.23	16.31	5.74
46	—20	803,000	610,000	563,000	100,000	28,000	11,500
Per cent viable	.....	100.00	75.97	70.11	12.45	3.49	1.43

—10°C., and 1.43 per cent in cider at —20°C. Greater survival of microbial forms in cider frozen and stored at —10°C. than in that at —20°C. has been previously reported by Berry and Diehl (1934).

Microbial flora were so diverse and pulp fragments so varied and numerous that the haemocytometer method of direct counting was not applicable in determining distribution of total cells. Therefore, yeast cells were suspended in commercial Seitz-filtered cider and the suspension was dispensed, frozen, and sectioned as described above for fresh cider. The haemocytometer counts made on the thawed sections showed that the distribution of total yeast cells in cider followed the same pattern as in 10-per cent sucrose, McFarlane (1940).

Macroscopic evidences of freezing redistribution are shown (Fig. 1).

FROZEN SYRUP-PACKED RASPBERRIES

Sorted but unwashed Cuthbert raspberries were packed in "malt quart" containers and sufficient 50.33-per cent cane sugar syrup was added to cover. Containers averaged 533 grams of raspberries and 326 grams of syrup. Viable microbial content at the time of freezing

was 575,000 per c.c. pulped berries and syrup. Most of the samples were frozen and stored in still air at —20°C.; a few were so handled at —10°C. From time to time, samples held at —20°C. were sectioned and the thawed sections examined for viable microörganisms, soluble solids, and hydrogen-ion concentration. Whole (unsectioned) samples held at —10 and —20°C. were examined periodically for viable microörganisms. One-c.c. semi-fluid portions of well-mixed pulped berries and syrup were diluted for plating purposes. Nutrient agar plates were incubated five days at 22°C.(71.6°F.).

The distribution of viable microbial forms during the first 12 weeks at —20°C. was not exactly uniform, although the tendency for such a distribution was indicated. While some sections in a given sample gave much higher counts than others, these sections were not constant in their position in all samples, a fact which suggested the presence in them of one or more heavily contaminated berries. After one week, section counts ranged from 95,000 to 1,450,000; after 26 weeks, from 26,000 to 672,000. Sectional studies made after the 16th week and on later dates showed that some slight change was occurring in the microbial picture besides the general decrease in viable forms in all sections. Specifically, this change consisted of a more rapid decrease of viable microörganisms in sections from the upper half of the sample.

Differences were not great in the soluble solid content of sections cut from frozen syrup-packed raspberries. Extremes ranged from 26.76 to 39.89 per cent. Bottom sections gave the highest refractometer readings. Sections from the lower half of the frozen mass were usually high in soluble solid content. Surface sections gave higher readings than the three or four sections immediately beneath. Sections which were higher in content of viable microörganisms at the end of the 26th week were generally the ones higher in soluble solid content. No orderly redistribution of hydrogen ions was noted. Sectional differences rarely exceeded .2 to .3 pH. There were only very slight macroscopic evidences of freezing redistribution (Fig. 2).

Since the raspberries naturally prevented the free passage of suspended minute particles, and also interfered with the concentration at any one point of considerable quantities of any substances in solution, a true redistribution of microörganisms, soluble solids, and hydrogen ions was not expected. Furthermore, the liquid medium, 50-per cent cane sugar syrup, although diluted with juice from the berries, still remained sufficiently concentrated to retard the activity of freezing and gravitational forces. The fact that there was not a great diversity of microbial behaviors exhibited in the different sections of a frozen mass of syrup-packed raspberries at —20°C. may

be attributed to the fact that there was not a wide variety of environmental conditions, particularly of extreme conditions.

There was a progressive decrease in the number of viable microörganisms in syrup-packed raspberries frozen and stored in still air at —10 and —20°C. After two weeks at —10°C. the count dropped

FIG. 2. Central vertical slab cut from syrup-packed raspberries frozen at —20°C.(—4°F.). (More syrup than usually present in bottom.)

to 478,000 (16.87 per cent) and after 24 weeks it dropped to 2,767 (99.52 per cent). Ninety-nine per cent decrease occurred at —10°C. some time between the 16th and 20th week. After freezing and storing two weeks at —20°C. the count dropped from 575,000 to 528,000 (8.17 per cent) and after 24 weeks it dropped to 141,000 (75.48

per cent). Greater decrease at the higher temperature in similarly packed berries has been reported by Berry (1932, 1933, 1936).

FROZEN BRINE-PACKED PEAS

"Malt quarts" were filled with blanched peas and the peas were covered with two-per cent sodium chloride brine, 627 grams of peas and 210 grams of brine per container. Samples were frozen and stored in still air at —20°C.(—4°F.); some were held at —10°C. (14°F.). Microbial count on a well-mixed sample of peas and brine prior to freezing was 1,050,000 microörganisms per c.c. of brine. Sectional studies, including one-per cent dextrose-agar plate counts, refractometer, and pH readings, were made periodically on samples frozen and stored at —20°C. One-cubic centimeter or fractional portions of the brine were used in plating and plates were incubated five days at 22°C.(71.6°F.). Whole samples, at —10 and at —20°C., were examined to compare microbial behavior at the two temperatures.

The viable microörganism count was high in all sections of a sample at the end of one week's storage. Bottom sections gave the highest counts. Surface sections gave higher counts than those immediately below. Lower core sections gave relatively low counts compared with counts given by corresponding sections from the intermediate and peripheral zones. Extremes in count ranged from 335,000 to 1,855,000. After 24 weeks the viable microbial content had decreased in all sections. Bottom sections still gave the highest counts and surface sections continued to give higher counts than the sections immediately below. Lower core sections, however, tended to give higher counts than corresponding intermediate or peripheral sections. Soluble solid distribution was greatest in bottom sections and in sections of the lower core and intermediate zones; surface sections were also high. Readings ranged from 4.90 to 9.93 per cent. Tabulated data indicated that the solids were concentrated in an inverted cone-shaped core and in and on the surface layer. The distribution pattern for viable microbial forms after the 24th week practically paralleled that for soluble solid distribution. No appreciable redistribution of hydrogen ions was observed. Sectional differences rarely exceed .2 pH. There was some macroscopic evidence that freezing forced the peas toward the center. In a centrally cut vertical section the peas had the appearance of having been forced away from the walls of the container and toward the center. Intervening fluid spaces in the core areas were smaller (Fig. 3).

Microbial counts on whole samples at —10 and —20°C. remained high throughout the 24-week storage period. Average plate counts

on three samples from each storage room were 724,000 and 515,000, respectively. These comparative counts and others made during earlier weeks suggested that the decrease in viable microörganisms tended to be greater at the lower temperature.

FIG. 3. Central vertical slab cut from brine-packed peas frozen at —20°C. (—4°F.). (About natural size.)

SUMMARY

In "malt quarts" (750 c.c.) of cider frozen and stored at —20°C. (—4°F.) microbial forms, soluble solids, and total (titratable) acids were concentrated in central cone-shaped areas and in surface areas. There was no marked freezing redistribution of microbial forms, soluble solids, or hydrogen ions in frozen syrup-packed raspberries

or brine-packed peas. Hydrogen ions were not appreciably redistributed in the three food products.

In frozen cider at —20°C. viable microörganisms decreased most rapidly in sections high in soluble solid content, while in samples at —10°C.(14°F.) the greatest number of viable cells after 31 weeks was found in the unfrozen concentrate which had approximately six times greater soluble solid and total acid content than normal cider. During the latter part of a 26-week storage period viable microbial counts in frozen raspberries at —20°C. were slightly greater in sections from the lower half. These sections were a little higher in soluble solid content. In frozen peas at —20°C. for 24 weeks, the viable microbial distribution tended to parallel the soluble solid distribution; i.e., tended to be concentrated in a central cone-shaped area and in a surface area.

In whole (unsectioned) samples greater decrease in viable microörganisms occurred in cider held at —20°C. than in cider held at —10°C. The reverse was true for syrup-packed raspberries. Large numbers of microbial forms remained viable for 24 weeks at both temperatures in brine-packed peas.

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EFFECT OF SOIL MOISTURE ON ACID CONTENT OF TOMATOES.[1] A PRELIMINARY REPORT

F. A. LEE AND C. B. SAYRE
New York State Agricultural Experiment Station,
Geneva, New York

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Several writers, Bigelow (1916, 1917), Sando (1920), Brooks and MacGillivray (1928), and Saywell and Cruess (1932), have suggested that rainfall might have some effect on the percentage of acid or other substances in the ripe tomato. Information of this sort should be of interest to manufacturers of tomato juice and of such concentrated products as tomato paste. In juice, especially, any variation from year to year in flavor of a given brand might be noticed by the consumer, making it desirable to avoid such differences if possible. This difference in flavor might be readily detected if the tomatoes used in the making of the juice one year had been harvested during a wet season, and the product the following year had been made from the same variety of tomatoes gathered during a very dry season.

The authors noticed over a period of several years that the acid content of tomatoes varied during the season and in dry and wet parts of the season. It was decided to verify this by running controlled experiments in the greenhouse with four varieties of tomatoes.

The four varieties used in this test represented distinct types and were chosen for specific reasons. John Baer and Marglobe are two of the principal varieties used commercially for canning and juice in New York state. King Humbert is a small, Italian-type tomato of low acidity used principally for tomato paste. Golden Queen is the principal variety used for yellow tomato juice. Claims have been made that the yellow varieties are less acid than red varieties. Apparently there is no basis for such claims, at least in this area, because two years' tests of 21 varieties of field-grown tomatoes at Geneva have shown that the yellow varieties are, in most cases, more acid than the red varieties; the higher acidity of the yellow variety (Golden Queen) is again shown in these tests (Tables 1 and 2). Duggar and Merrill (1914) reported acid content of the yellow and red varieties to be the same.

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[1]Approved by the director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 335, June 9, 1939.

TABLE 1

Effect of Soil Moisture on Percentage of Acids and Total Solids in Ripe Tomatoes

(Fresh-Weight Basis)

Harvest dates	King Humbert				John Baer				Supreme Marglobe				Golden Queen			
	Wet		Dry		Wet		Dry		Wet		Dry		Wet		Dry	
	Acid	Solids	Acid	Solids	Acid	Solids	Acid	Solids	Acid	Solids	Acid	Solids	Acid	Solids	Acid	Solids
1938	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*
June 1	.....	.....	.66	.....	.....	.....	.59	.....	.....	.....	.....	.....	.....	.....	.....	.....
June 6	.....	.....	.58	.....	.....	.....	.68	.....	.66	.....	.....	.....	.....	.....	.84	.....
June 9	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.74	.....	.....	.....	.81	.....
June 11	.....	.....	.75	.....	.....	.....	.66	.....	.....	.....	.60	.....	.45	.....	.....	.....
June 18	.37	.....	.74	8.31	.43	.....	.56	.....	.47	.....	.56	7.70	.44	.....	.74	.....
June 22	.32	6.25	.62	9.66	.43	6.37	.60	7.62	.50	5.89	.61	7.48	.35	5.67	.78	9.81
June 27	.....	.....	.46	9.37	.47	6.03	.....	.....	.57	6.96	.....	.....	.41	6.20	.62	10.13
June 28	.26	6.30	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
June 30	.21	6.25	.....	.....	.33	6.15	.37	6.75	.....	.....	.....	.....	.....	.....	.....	.....
July 2	.21	6.41	.39	9.58	.....	.....	.38	6.73	.32	6.26	.....	.....	.32	6.90	.48	.....
July 6	.....	.....	.....	.....	.34	7.54	.36	7.28	.39	7.25	.30	7.51	.....	.....	.....	.....
July 8	.20	6.17	.....	.....	.27	7.05	.....	.....	.38	7.21	.....	.....	.25	6.66	.....	.....
July 9	.....	.....	.43	9.94	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
July 11	.18	5.88	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.31	7.57	.....	.....
July 13	.31	6.93	.55	11.34	.....	.....	.50	7.49	.....	.....	.46	6.68	.39	6.16	.71	8.91
July 15	.17	6.80	.....	.....	.25	6.97	.....	.....	.28	7.40	.....	.....	.....	.....	.....	.....
July 18	.32	6.87	.52	10.16	.43	7.95	.57	8.10	.63	8.61	.57	7.89	.48	8.47	.62	8.61
Av. acid for season	.26	.....	.57	.....	.37	.....	.53	.....	.47	.....	.55	.....	.38	.....	.70	.....

EXPERIMENTAL PROCEDURE

For this test 10 plants of each variety were grown in Ontario loam soil on a greenhouse bench which was watered frequently to maintain a high soil-moisture content and approximate conditions of abundant rainfall. A similar lot of each variety was grown on an adjoining bench receiving very limited water and approximating conditions of drought.

Samples of the fruit grown under both conditions were collected and analyzed throughout the growing period. Composite samples of

TABLE 2

Effect of Soil Moisture on Percentage of Acid in Ripe Tomatoes
(Dry-Weight Basis)

Harvest dates	King Humbert		John Baer		Supreme Marglobe		Golden Queen	
	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
1938	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*
June 18	.....	8.90	.....	.....	.....	7.27	.....	.....
June 22	5.12	6.42	6.75	7.87	8.49	8.16	6.17	7.95
June 27	.....	4.92	7.82	.....	8.19	.....	6.61	6.12
June 28	4.13	.....	.....	.....	.....	.....	.....	.....
June 30	3.36	.....	5.37	5.48	.....	.....	.....	.....
July 2	3.28	4.14	.....	5.65	5.11	.....	4.64	.....
July 6	.....	.....	4.48	4.95	5.38	4.00	.....	.....
July 8	3.24	.....	3.83	.....	5.27	.....	3.75	.....
July 9	.....	4.33	.....	.....	.....	.....	.....	.....
July 11	3.06	.....	.....	.....	.....	.....	4.10	.....
July 13	4.48	4.85	.....	6.67	.....	6.88	6.33	7.91
July 15	2.50	.....	3.59	.....	3.78	.....	.....	.....
July 18	4.66	5.12	5.41	7.04	7.32	7.23	5.67	7.20
Average	3.76	5.53	5.32	6.28	6.22	6.71	5.32	7.30

several fruits were used for each analysis. Total solids were determined by heating at 95°C.(203°F.) for 48 hours, and acid was titrated directly according to the method of the American Association of Agricultural Chemists. It is perhaps needless to say that the tomatoes grown under conditions of drought were firmer and smaller than those grown under more favorable conditions.

The percentage of total acid calculated as citric acid and the percentage of total solids on a fresh-weight basis are shown (Table 1). This table shows the variations in acidity throughout the harvesting period and the average acidity of each variety for the entire season. Acid content on the fresh basis is important in the case of tomatoes to be used for the manufacture of juice, because the product resulting from the juicing operation is canned directly without concentration.

The same results calculated on a dry-weight basis are included (Table 2). The results obtained for the first few days of the experiment could not be so calculated because the total-solids figures were not determined. These data show that the larger figures for total acid in the fruits under drought conditions are actual and not merely a larger percentage caused by the smaller amounts of water in those plants grown under dry conditions. The canning-type varieties (John Baer and Marglobe) showed less variation in acidity owing to soil moisture than the other varieties. The yellow variety (Golden Queen) showed the highest acidity and also the greatest increase in acidity under drought conditions. The paste-type variety (King Humbert) contained the least acid but showed a marked increase in acidity when the soil moisture was limited.

DISCUSSION OF RESULTS

It can be seen from the tables that the acid content of the ripe fruit at the beginning of the season is high, then it drops in the fruit harvested as the season progresses, and finally is high again in the ripe fruit at the end of the season, when grown under dry or moist conditions.

Since the acid content of tomatoes is an important factor in the flavor of any product made from them, it is apparent that this will have to be watched if uniform products are to be obtained from year to year; and while acid can be neutralized, it might be more desirable to select varieties that show a minimum of variation in acid content when grown under different moisture conditions. A number of commercially important varieties are being studied to obtain this information about them.

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TYPES OF ORGANISMS SURVIVING IN COMMERCIALLY PASTEURIZED CITRUS JUICES IN FLORIDA[1]

ARTHUR J. NOLTE AND HARRY W. VON LOESECKE

U. S. Citrus Products Station, Bureau of Agricultural Chemistry and Engineering, U. S. Department of Agriculture, Winter Haven, Florida

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Although citrus juices (primarily grapefruit) have been canned in Florida for many years by exhausting, sealing, and then processing, this method is now being replaced by the more modern technique of high-short[2] pasteurization. Whereas the old method subjected the juice to temperatures of 80 to 85°C.(176 to 185°F.) for periods ranging from one-half to one hour, in the high-short procedure the juice is heated at 85 to 88°C.(185 to 190°F.) for only a few seconds. It has been found that this method, especially when used in conjunction with efficient de-aeration, aids in preserving the natural aroma and taste of the juice and its vitamin C potency and prolongs storage life. These temperatures and times, too, are probably sufficient to inactivate enzymes responsible for subsequent settling or "curdling" of the processed juice.

A bacteriological study of organisms present in fresh citrus juices and those surviving high-short pasteurization should be of definite practical value. Pathogenic organisms of the colon-typhoid-dysentery group are said to be found on citrus fruits and in their juices, Garcia (1911). Possible sources of such contamination may include flies, picking and handling, polluted wash waters, and the use of drops. These pathogens and indicators of pathogens would not survive pasteurization temperatures in the case of citrus juices because of the acidity of the medium. For this reason, microörganisms in citrus juices are more easily destroyed by high-short pasteurization than microörganisms in milk, Breed (1932).

According to Berry (1932), thermophilic and similar heat-resistant organisms have been found in canned tomato juice, giving the juice an off-flavor which was almost nauseating in extreme cases. In the present work thermophiles were isolated from pasteurized citrus juices. These organisms were plated out on dextrose agar and incubated at 55°C.(131°F.) under aerobic and anaerobic conditions.

[1] Food Research Division Contribution No. 438.

[2] This is the term suggested by Shrader as a substitute for "flash." This latter term is considered a misnomer by most food technologists.

TABLE 1

Bacteriological Examination of Raw and Pasteurized Canned Citrus Juice—Plant A

(Organisms per c.c.)

Sampling date	Wort agar	Nutrient agar				Tomato-juice agar		Trypsin-digest agar	
		Aerobes 37°C. (98.6°F.)	Anaerobes 37°C.	Aerobes 20°C. (68°F.)	Anaerobes 20°C.	Aerobes 37°C.	Anaerobes 37°C.	Aerobes 37°C.	Anaerobes 37°C.
Raw Juice									
January 7, 1938	119,700	87,000	....	58,900	....	....	....	....	....
January 17, 1938	1,900	1,800	....	2,500	....	....	....	....	....
February 4, 1938	13,000	39,700	....	10,000	....	....	....	....	....
February 19, 1938	44,000	37,000	....	44,000	....	47,000	....	11,000	....
March 10, 1938	354,000	438,000	....	180,000	....	744,000	....	657,000	....
April 15, 1938	514,600	106,000	91,000	217,800	121,000	371,000	272,800	316,000	208,000
Pasteurized Canned Juice									
January 7, 1938	0	0	....	0	....	....	....	....	....
January 17, 1938	0	0	....	0	....	....	....	....	....
February 4, 1938	0	0	....	0	....	....	....	....	....
February 19, 1938	0	0	....	0	....	0	....	0	....
March 10, 1938	5	1	....	2	....	0	....	0	....
April 15, 1938	0	0	0	0	0	0	0	1	1

EXPERIMENTAL PROCEDURE

Samples of juice from the same lots before and after processing were obtained at different citrus canneries. Fresh juice was sampled immediately as it came from the extractors or was taken from blending tanks. The pasteurized juice was obtained either from the filler bowl or from sealed cans after they had passed through the cooling tanks. The samples were plated within 30 minutes after they had been obtained.

Plating was carried out on nutrient agar, wort agar, trypsin-digest agar, and tomato-juice agar. For organisms of the coli-aerogenes

TABLE 2

Bacteriological Examination of Raw and Pasteurized Canned Citrus Juice—Plant B[1]

(Organisms per c.c.)

Sampling date	Wort agar	Nutrient agar		Tomato-juice agar 37°C.	Trypsin-digest agar 37°C.
		37°C. (98.6°F.)	20°C. (68°F.)		
Raw Juice					
January 7, 1938	18,600	2,000	25,000	....	....
January 17, 1938	800	800	3,400	....	....
February 4, 1938	17,000	13,000	48,100	....	....
February 19, 1938	22,000	31,000	29,000	28,000	16,000
March 17, 1938	14,000	6,000	35,000	2,000	9,000
Pasteurized Canned Juice					
January 7, 1938	0	11	2	....	....
January 17, 1938	0	0	0	....	....
February 4, 1938	0	0	0	....	....
February 19, 1938	0	0	20	1	0
March 17, 1938	0	0	0	0	0

[1] Counts are all aerobic.

group, lactose broth buffered with potassium acid phosphate was used and incubated according to the methods of the American Public Health Association (1933). Results from these tests were confirmed by Endo's agar and Brilliant Green Lactose Bile Agar with reinoculation of picked colonies from Endo's agar into Lactose broth. With the exception of wort-agar plates, incubation was at 37°C. (98.6°F.) for 72 hours. Wort-agar plates were incubated at 25°C. (77°F.) for 72 hours; and some of the counts on nutrient agar were made at 20°C. (68°F.). Anaerobic counts were accomplished according to Dick's anaerobic plate method, used by Tanner (1928), in which dilutions were plated as usual, an additional layer of agar being poured over the hardened agar in the plate. After cooling, sterile melted paraffin

TABLE 3

Bacteriological Examination of Raw and Pasteurized Canned Citrus Juice—Plant C

(Organisms per c.c.)

Sampling date	Wort agar	Nutrient agar				Tomato-juice agar		Trypsin-digest agar	
		Aerobes 37°C. (98.6°F.)	Anaerobes 37°C.	Aerobes 20°C. (68°F.)	Anaerobes 20°C.	Aerobes 37°C.	Anaerobes 37°C.	Aerobes 37°C.	Anaerobes 37°C.
Raw Juice									
January 7, 1938	44,000	40,100	....	62,000	....	....	....	....	....
January 17, 1938	11,600	14,300	....	15,000	....	....	....	....	....
February 4, 1938	121,000	89,000	....	136,400	....	....	....	....	....
February 19, 1938	92,000	106,000	....	236,000	....	125,000	....	98,000	....
March 10, 1938	87,500	38,000	....	26,000	....	58,000	....	37,000	....
March 24, 1938	123,500	73,000	....	160,000	....	76,000	....	130,000	....
April 13, 1938	....	....	11,160	....	31,620	....	72,540	....	37,450
April 28, 1938	59,000	58,000	16,000	85,000	75,000	80,000	85,000	15,500	84,500
Pasteurized Canned Juice									
January 7, 1938	0	0	....	0	....	....	....	....	....
January 17, 1938	0	0	....	0	....	....	....	....	....
February 4, 1938	0	5	....	0	....	....	....	....	....
February 10, 1938	0	0	....	0	....	0	....	1	....
March 10, 1938	0	0	....	0	....	0	....	1	....
March 24, 1938	0	....	....	0	....	0	....	0	....
April 13, 1938	0	....	0	....	1	....	0	....	0
April 28, 1938	0	0	0	0	0	0	0	0	0

was poured on this second coating of agar. Plates were then placed in a Novy jar and the jar exhausted.

DISCUSSION OF RESULTS

All of the raw-juice samples showed yeasts, molds, and bacteria in considerable numbers (Tables 1, 2, 3, 4, 5, and 6). Variations in counts could not be correlated with the apparent cleanliness of the plant, nor was there any significant difference in the counts of samples taken periodically during the season. Camp and Stahl (1934), in one of their papers, report a considerably lower count for freshly extracted orange juice than was obtained by the authors. Their juice was prepared in the laboratory, but without special precautions as to sterility of equipment. It would seem that the counts reported here for samples representing commercial extraction of citrus juices are unnecessarily high and undoubtedly could be lowered by the use of chlorine after the fruit has passed through the washers.

In only one sample of pasteurized grapefruit juice was a mold count obtained. This sample was taken from a can collected at the start of the day's run at one plant.

A number of colonies of yeasts and molds from raw juice were obtained from the wort-agar plates for subcultures. These were examined and identified as follows:

Saccharomyces cerevisiae	*Saccharomyces ellipsoideus*
Saccharomyces apiculatus	*Saccharomyces pastorianus*
Torula (one a pink torula) *sp.*	*Mycoderma cerevisiae*
Dematium sp.	*Oidium* (one a red oidium) *sp.*
Monila sp.	*Penicillium glaucum*
Aspergillus glaucus	*Aspergillus sp.*

Over 100 cultures of bacteria were isolated from the raw juice. No attempt was made to identify the organisms in the raw juice because it was felt the time involved to carry out a complete study would not be justified. It was considered more important to attempt the identification of those organisms surviving the high-short pasteurization.

None of the bacteria isolated from pasteurized juice would grow on orange or grapefruit juice agars under either aerobic or anaerobic conditions at 25, 37, and 55°C. (77, 98.6, and 131°F.). Some of the isolated organisms did, however, show growth on dextrose agar at 55°C. The inability of all the organisms isolated in our study, including the thermophiles, to grow in citrus juices indicates that the organisms are not a factor in the development of off-flavors in canned juice. The molds isolated, however, did grow under aerobic conditions at 25°C.

TABLE 4

Bacteriological Examination of Raw and Pasteurized Canned Citrus Juice—Plant D

(Organisms per c.c.)

Sampling date	Wort agar	Nutrient agar				Tomato-juice agar		Trypsin-digest agar	
		Aerobes 37°C. (98.6°F.)	Anaerobes 37°C.	Aerobes 20°C. (68°F.)	Anaerobes 20°C.	Aerobes 37°C.	Anaerobes 37°C.	Aerobes 37°C.	Anaerobes 37°C.
Raw juice, May 5, 1938	165,000	56,000	53,000	102,000	35,000	117,000	109,000	167,000	79,000
Canned juice, May 5, 1938	0	6	42	30	18	2	3	7	4

TABLE 5

Bacteriological Examination of Raw and Pasteurized Canned Citrus Juice—Plant E

(Organisms per c.c.)

Sampling date	Wort agar	Nutrient agar				Tomato-juice agar		Trypsin-digest agar	
		Aerobes 37°C. (98.6°F.)	Anaerobes 37°C.	Aerobes 20°C. (68°F.)	Anaerobes 20°C.	Aerobes 37°C.	Anaerobes 37°C.	Aerobes 37°C.	Anaerobes 37°C.
RAW JUICE									
April 15, 1938	32,000	26,000	25,000	155,000	110,000	21,000	18,000	25,000	15,000
May 28, 1938	24,000	21,000	13,000	36,000	5,000	77,000	34,000	42,000	55,000
PASTEURIZED CANNED JUICE									
April 15, 1938	0	19	5	11	6	3	2	5	2
May 28, 1938	0	1	3	1	0	1	0	0	0

Survival and destruction determinations of organisms isolated from pasteurized juice were made by inoculating sterile orange juice (pH 3.7) with a loop of a six-day culture of the organism. The inoculated juice was contained in Pyrex test tubes which were placed in a water bath heated to 85°C.(185°F.). A similar tube of orange juice with a thermometer was used as control for temperature readings. One minute and two minutes after the juice had reached the desired temperature a loop was removed from the inoculated tube and streaked on nutrient agar. At intervals of two minutes additional loops of material were withdrawn. A temperature of 85°C. was selected as it is the minimum temperature of pasteurization usually employed in Florida citrus canneries. The other temperature used in these tests was 99°C.(210.2°F.). Although the tubes were in

TABLE 6

Presumptive Coli-Aerogenes Lactose-Broth Tests of Raw Citrus Juice, and Water From Fruit-Washing Tanks[1]

(Organisms per c.c.)

Sampling date	Plant A[2]	Plant B	Plant C[3]
January 7, 1938	<1	<1	0
January 17, 1938	<1	<1	<1
February 4, 1938	1,000	1,000	0
February 17, 1938	100	100	55
March 17, 1938	1	0	1

[1] Quantitative counts computed according to "Standard Methods of Water Analysis." 7th ed. p. 132(b) (1933). [2] Fruit-washing tank water, Plant A—10,000 on February 17, 1938. [3] Fruit-washing tank water, Plant C—1,000 on February 4, 1938.

a boiling water bath, the contents never actually reached 100°C. (212°F.).

Survival and destruction times, determined as described above, of unidentified organisms from pasteurized citrus juices are given (Table 7).

The destruction time of most of the organisms surviving high-short pasteurization is sufficiently long to prevent their destruction by this method of pasteurization. Their inability to grow in orange or grapefruit juice, however, renders them unable to cause spoilage from a biological point of view.

Organisms isolated from pasteurized juice and identified consisted of the following[3]: *Bacillus mesentericus, Bacillus subtilis, Bacillus vulgatus, Mucor sp.* and *Penicillium glaucum* (these two molds were isolated from one of the first cans from the line when the day's run

[3] Identified and named according to Bergey's "Manual of Determinative Bacteriology." 4th ed. (1934).

was started), *Bacillus cylindricus* (or *Bacillus cytaseus*), *Lactobacillus thermophilus*, *Bacillus non diastaticus*, *Bacillus cereus*, and *Sarcina sp.*

SUMMARY

Samples of fresh and high-short commercially pasteurized citrus juices (orange and grapefruit) were taken at intervals during the canning season from five different canneries for bacteriological examination.

The yeast and mold count of raw juice ranged from a minimum of 800 to a maximum of 514,600 per c.c. Bacterial count (aerobic) ranged from a minimum of 800 to a maximum of 744,000 per c.c.

Variation in counts could not be correlated with the apparent cleanliness of the plant, nor was there any significant difference in counts of samples taken periodically during the season.

TABLE 7

Survival and Destruction in Orange Juice (pH 3.7) of Organisms (Unidentified) Isolated From Pasteurized Citrus Juices[1]

Organism No.	Survival and destruction times
1, 15, 21, 23	Survived 10 min. at 99°C.(210.2°F.)
4	Destroyed in 7 min. at 99°C.
8, 19, 14	Destroyed in 5 min. at 85°C.(185°F.)
10	Destroyed in 1 min. at 99°C.
16	Destroyed in 3 min. at 85°C.

[1] See text for method of determination.

Coli-aerogenes group of organisms showed a count ranging from a minimum of zero to a maximum of 1,000 per c.c. in raw juice.

Escherichia coli was shown to be present in raw juice but absent in pasteurized juice.

Water from washing tanks in the two canneries sampled showed high coli-aerogenes content.

Bacterial count of pasteurized juice showed a minimum of zero and a maximum of 30 per c.c. under aerobic conditions; and a minimum of zero to a maximum of 42 per c.c. under anaerobic conditions. None of the bacteria isolated from the pasteurized juice would grow on orange or grapefruit agars either aerobically or anaerobically. Some, however, grew on dextrose agar at 55°C.(131°F.).

Survival and destruction times of most of the organisms not destroyed by high-short pasteurization were sufficiently high to prevent their destruction by this means of pasteurization. Their inability to grow in orange or grapefruit juice, however, renders them unable to cause spoilage from a biological point of view.

High-short pasteurization of orange and grapefruit juice as practiced in the canneries studied is shown to be efficient in destroying organisms which may later cause biological spoilage.

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CARCASS STUDIES ON SOME GRAIN-FED AND GARBAGE-FED HOGS

E. H. HUGHES AND N. R. ITTNER
Division of Animal Husbandry, College of Agriculture, University of California, Davis, California

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An appreciable amount of pork in California and certain other states comes from hogs fed garbage at some time during their lives. It is also true that hogs almost everywhere are fed kitchen waste. In so far as nutrients are concerned, fresh garbage should be a reasonably well-balanced diet for the pig, except for the fact that the capacity of the pig for food consumption is limited and that garbage is bulky. In most places, however, the pork produced from garbage is discriminated against because of the belief that such pork is inferior in quality. In an effort to determine whether pork from garbage-fed hogs differs from that of grain-fed hogs, the carcasses from both kinds have been graded; the refractive index of the fat has been determined; the moisture content of the muscle and fat has been examined; and the sodium chloride content of the bacon from each has been studied.

EXPERIMENTAL PROCEDURE

The information reported herein is from garbage-fed hogs from San Francisco and Los Angeles, and grain-fed hogs from northern and southern California. The grain-fed hogs were given such feeds as barley, wheat, tankage, fish meal, linseed meal, wheat middlings, and skim-milk with and without pasture. Many carcasses were graded and the refractive index of the fat was determined for nearly 600 carcasses. Carcasses were examined from hogs fed grain without garbage, those self-fed grain with what garbage they would consume in addition, those given one feed of grain each day with all the garbage they would consume the rest of the day, and those fed only garbage.

GRADING AND REFRACTIVE-INDEX VALUES

Two measures of firmness or hardness of the carcasses were employed: (1) physical grading of the carcasses after a 48-hour storage in the cooler; and (2) the refractive index of the rendered fat. These are the standard measurements used for such studies. The refractive index was originally adopted by workers in this field because it could be determined rapidly and has given satisfactory results.

TABLE 1

Results of Grading Carcasses in Cooler at about 2.2°C.(36°F.)

Group No.	Rations fed	Grading results				
		Hard	Medium hard	Medium	Medium soft	Soft
1	Grain, or grain self-fed plus garbage	145	81	38	26	7
2	Garbage, or grain fed once a day plus what garbage they would consume	16	24	37	26	4

TABLE 2

Refractive-Index Values of Back Fat of Carcasses

Group No.	Rations fed	Refractive-index values					
		1.4569 and below	1.4570 to 1.4579	1.4580 to 1.4589	1.4590 to 1.4599	1.4600 to 1.4609	1.4610 and above
1	Same as Group 1, Table 1	12	32	177	180	23	8
2	Same as Group 2, Table 1	0	5	15	55	61	21

TABLE 3

Moisture Content of Muscle Before and After Processing[1]

Group No.	Number of samples	Ration fed	Moisture percentage	
			Before processing	After processing
1	9	Grain	68.2	55.9
2	9	Grain self-fed, plus garbage	68.1	51.6
3	10	Grain fed once a day, plus what garbage they would consume	70.1	57.0
4	10	Garbage only	70.1	52.3
Average of 38 samples...		.....	69.1	54.2

[1] This was from bacon cured and smoked by one of the large packers.

TABLE 4

Sodium Chloride Content of Muscle and Fat of Bacon After Processing[1]

Group No.	Number of samples	Ration fed	Sodium chloride percentage	
			Muscle	Fat
1	9	See Table 3	11.8	1.2
2	9	See Table 3	13.0	1.3
3	10	See Table 3	13.0	1.4
4	10	See Table 3	13.4	1.2
Average of 38 samples..		.....	12.8	1.3

[1] This was from bacon cured and smoked by one of the large packers.

In examining the data the results of grading by a committee of three in the cooler and the refractive-index values were so similar for hogs fed grain and for those self-fed grain with what garbage they would consume that they have been grouped together. The results of those given one feed of grain each day with garbage *ad libitum*, and those fed only garbage, were similar, and have been grouped (Tables 1 and 2).

MOISTURE CONTENT OF MUSCLE AND FAT BEFORE AND AFTER PROCESSING AND SALT CONTENT OF EACH IN CURED AND SMOKED BACON

The fresh muscle used was taken from the inner facing of the ham, while the fat was back fat from above the 5th to 7th ribs. The muscle and fat studied from the processed product was taken from the belly. For chemical analysis both the muscle and fat were quickly cut into small cubes and dried in an electric oven at 90°C. (194°F.). After the determination of moisture was made, the residue was ashed and the ash taken up in distilled water. Chloride was determined in this solution, using the volumetric method with standard silver nitrate and sodium chromate indicator.

Most of the data under this caption is shown (Tables 3, 4, and 5); however, the moisture content of the fat was very difficult to secure and only a limited number of determinations were made. The average water content for seven samples determined before processing was 6.9 per cent and for five samples from cured and smoked bacon was 6.9 per cent, showing no difference in the average before and after. There was considerable variation, however, even in the limited number of determinations made.

DISCUSSION OF RESULTS

A significant difference is noticeable in the grading results and in the refractive-index values of the fats of the two groups, the grain-fed carcasses being considerably harder than those given garbage (Tables 1 and 2). Hankins and Ellis (1926) considered carcasses hard when the back fat had a refractive index of 1.4597 or below. On this basis 84 per cent of the carcasses in Group 1 and only 35.7 per cent of those in Group 2 were hard. Apparently there were enough soft fats and oils in the garbage to account for the difference.

There seemed to be no significant difference in the moisture content of the fat or muscle between those given grain and those fed garbage either in the fresh or the processed muscle. There was, however, a difference of about 15 per cent in the water content of fresh and processed muscle (Table 3).

A difference is evident in the salt content of the processed muscle from grain and garbage carcasses (Groups 1 and 4, Table 4). The sodium chloride in the fat for the various groups is similar and the percentage content is low, as would be expected. The average salt content for 38 samples of muscle in these studies was 12.9 per cent, which is lower than that reported by Miller and Ziegler (1939) for commercial hams and higher than that for their own cured hams. The difference between the salt content of the grain and garbage muscle (Groups 1 and 4) seems significant. Of the nine samples from the grain-fed carcasses, only two were above 12 per cent, while seven of the 10 samples of the muscle from garbage-fed hogs were

TABLE 5

Refractive-Index Values of Fat From Some of the Same Carcasses Before and After Processing

Group No.	Ration fed	Before processing		After processing[1]	
		Number of samples	Average refractive-index value	Number of samples	Average refractive-index value
1	See Table 3	24	1.4596	10	1.4593
2	See Table 3	24	1.4597	10	1.4596
3	See Table 3	24	1.4601	10	1.4604
4	See Table 3	24	1.4602	10	1.4604

[1] Only 10 samples of the original 24 were taken after processing. These were values from fat samples taken from bacon cured and smoked.

above 12 per cent. As measured by tasting, the cooked bacon from the garbage hogs was more salty.

While there was a considerable loss in the moisture content of muscle during curing and smoking (Table 3), this was compensated by the addition of salt during the process. As was expected, there was no change in the refractive-index values of the fat after the carcasses were cured (Table 5).

SUMMARY

By means of two measurements, physical grading of the carcasses in the cooler at a temperature of about 2.2°C.(36°F.) 48 hours after slaughter and by refractive-index values, it was found that the fat from the carcasses of hogs given grain was firmer than from similar carcasses of garbage-fed hogs.

The water content of the muscle from cured and smoked muscle from grain-fed hogs was lower than for similar muscle of hogs given garbage.

There was a loss of about 15 per cent in the moisture content of muscle during curing and smoking.

The average refractive-index values of the fat were the same before and after processing.

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EFFECTS OF NARINGIN AND HESPERIDIN ON ALBINO RATS[1]

ROBERT H. WILSON AND FLOYD DEEDS
Food Research Division, Bureau of Agricultural Chemistry and Engineering, U. S. Department of Agriculture, at the Department of Pharmacology, Stanford University School of Medicine, San Francisco, California

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Two glucosides, naringin and hesperidin, are present in appreciable quantities in the American diet. Naringin, according to Asahina and Inubuse (1929), when dried at 110°C.(230°F.), has the formula $C_{27}H_{32}O_{14}\cdot 2H_2O$ and melts at 171°C.(339.8°F.). When recrystallized from water it has an additional six molecules of water of crystallization and melts at 83°C.(181.4°F.). Naringin crystallizes as light yellow prisms and is soluble in hot water or hot alcohol but insoluble in ether. Upon acid hydrolysis naringin yields glucose, rhamnose, and naringenin (Pulley and von Loesecke [1939]). Naringin has an extremely bitter taste and is largely responsible for this property of grapefruit. The popular use of grapefruit as the first course of a meal, particularly breakfast, may depend in large measure upon the bitterness of naringin and its action as a stomachic.

Hesperidin is the corresponding glucoside present in oranges. In sharp contrast with naringin, it is practically tasteless. Asahina and Inubuse (1929) assign the formula $C_{28}H_{34}O_{15}$, and state that the products of acid hydrolysis are hesperetin, glucose, and rhamnose. When pure, it crystallizes in white, microscopic needles; the sample used in this study had a yellowish color. Hesperidin decomposes at 251°C.(483.8°F.), is slightly soluble in water and alcohol, but is insoluble in ether.

Need for a study of these substances is based upon several factors. Not infrequently plant glucosides have important pharmacological properties, and the two glucosides under consideration are consumed in fairly large quantities in the American diet. According to recent reports by Rusznyák and Szent-Györgyi (1936), Jersild (1938), and Scarborough and Stewart (1938) hesperidin, or "citrin," containing hesperidin, is vitamin P, which is claimed to be an important factor in controlling capillary permeability. While these claims have not been universally corroborated, Peters and Davenport (1938) state it is "unwise to pronounce sweepingly against the existence of a capillary factor for man." Of little scientific value, but nevertheless interesting, are the persistent verbal opinions that narin-

[1] Food Research Division Contribution No. 444.

gin is capable of lowering blood sugar, and that the ingestion of an infusion prepared by macerating an entire grapefruit and boiling with water is effective in the treatment of colds. If this is so, naringin and hesperidin would appear to be pharmacologically active; therefore it seemed worth while to make some general observations for actions on young growing rats, including evidences of visible injurious effects. As a first step in the pharmacological study of these two glucosides, feeding experiments were conducted on albino rats to determine the effects on growth and food consumption. The latter might indicate effects on appetite in the case of a bitter stomachic. Observations were also made on the blood-sugar level with naringin.

Only two reports were found which deal directly or indirectly with the effects of naringin and hesperidin on the animal organism. Garino (1913) gave .8 gram of naringin to a dog by mouth with entirely negative results, and naringin could not be detected in the urine. He injected hesperidin intraperitoneally and intravenously in dogs and isolated hesperidin, but not hesperetin, from the urine. After injecting 1.5 grams of hesperidin in one dog, Garino recovered one gram of unchanged hesperidin. Hesperetin, injected intravenously, was excreted unchanged in the urine, since hesperetin was identified, while phloroglucinol, one of its degradation products, was not demonstrable.

Although the investigation was conducted for another purpose, Willimott and Wokes (1926) actually contributed information on the chronic toxicity of naringin. These authors fed grapefruit rind, or an alcoholic extract of it, containing naringin, to rats on diets free of vitamin A or vitamin B. The rats on the vitamin B-free diet grew perfectly well when the rind or its extract was added to the diet. This was not true of the rats on the vitamin A-free diet plus grapefruit rind or its extract, but the addition of cod liver oil allowed complete recovery. The organs of all rats examined at autopsy were found to be normal, thus indicating that the prolonged feeding of naringin was not injurious.

EXPERIMENTAL PROCEDURE

In the present studies the samples of naringin and hesperidin used were supplied by the Los Angeles Laboratory of Fruit and Vegetable Chemistry of the Bureau of Chemistry and Soils, U. S. Department of Agriculture. The method used in feeding these two glucosides, and the stock diet employed, were the same as those used in previous studies by Wilson and DeEds (1936) on chronic intoxication. Male albino rats averaging 50 grams body weight at the start of the feeding experiment were used, six to eight rats for each dosage

level. The glucosides were mixed with the stock diet in concentrations up to one per cent by weight. The animals had free access to food and water at all times.

Naringin, particularly in a concentration of one per cent, gave the diet a noticeably bitter taste, but despite this the food was not rejected by the rats. After 150 days on the experimental diets, the weights of all rats on all levels of both naringin and hesperidin were within the normal limits for the control animals. During the next 50 days the growth curves were complicated by respiratory infec-

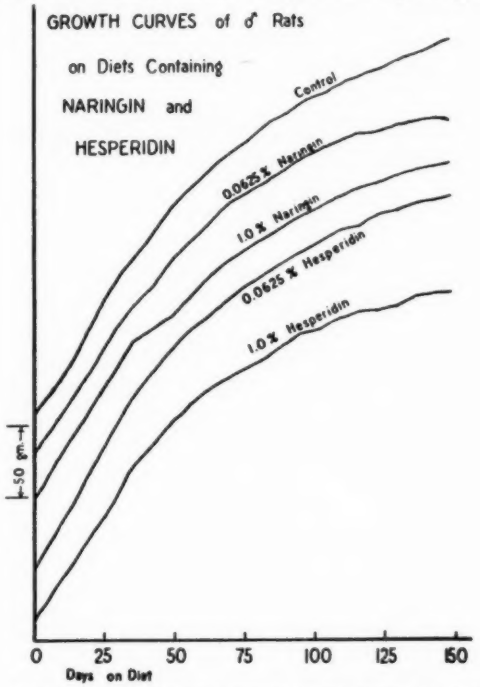

FIG. 1. Effects of naringin and hesperidin on growth of albino rats.

tions general throughout the laboratory colony. Although the data obtained do not permit statistical evaluation, the impression was gained that the incidence of respiratory distress was slightly less in those animals on the higher levels of naringin and hesperidin intake. Certainly the rats were no more susceptible to the infection than the controls or those on the lower glucoside dosages. The close parallelism of growth curves for control rats and those on the lowest and highest concentrations of naringin and hesperidin is shown (Fig. 1). In conformity with the close parallelism of these growth

curves is the fact that there were no significant differences in food intake between the control rats and those receiving naringin and hesperidin.

After the rats had been on the diets two months, blood-sugar determinations were made on the controls and on those receiving the highest concentration of naringin. In no case was a blood-sugar value found which was outside the range in normal animals. Thus, the reputed hypoglycemic action of this glucoside was not confirmed.

At the end of 200 days all the rats were killed with ether and autopsied. The weights of the hearts, spleens, livers, adrenals, kidneys, and testes were all within the normal range. All organs appeared normal macroscopically except the lungs of rats having respiratory infection. Histological examination of paraffin sections stained with hematoxylin-eosin showed no significant morphological changes in the livers, hearts, kidneys, spleens, adrenals, and testes of rats receiving hesperidin. Tissues of animals receiving naringin were not examined histologically.

SUMMARY

The continued feeding to healthy albino rats of naringin and hesperidin in a standard diet for a period of 200 days, in concentrations as high as one per cent of the diet by weight, gave no evidences of cumulative injury as judged by growth curves, blood-sugar levels, weights of important viscera, and macroscopic and microscopic examination of these tissues.

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DETERMINATION OF CAROTENE IN FRESH AND FROZEN VEGETABLES[1, 2]

I. CAROTENE CONTENT OF GREEN SNAP BEANS AND SWEET CORN

WILLIAM I. ZIMMERMAN AND DONALD K. TRESSLER
New York State Agricultural Experiment Station, Geneva, New York
AND
LEONARD A. MAYNARD
Animal Nutrition Department, Cornell University, Ithaca, New York

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PRELIMINARY STUDIES

In preliminary investigations of the carotene content of fresh and frozen vegetables by Stimson, Tressler, and Maynard (1939), the Russell, Taylor, and Chichester (1935) colorimetric method of determining carotene was found to be satisfactory for the estimation of this vitamin A precursor in peas. When, however, the method was applied to yellow sweet corn, Stimson (1939), the results were much higher than those obtained by bioassay of the same material. From this result it was concluded that either the procedure did not separate all of the biologically inactive yellow pigments from the beta carotene or the carotene of sweet corn was largely alpha or gamma carotene or cryptoxanthin, all of which possess only one-half the biological activity of beta carotene. Further, both postulations might be correct.

On the other hand, when the method was applied to fresh green beans the results obtained were absurdly low. Further, scalded beans apparently contained many times more carotene than the fresh vegetable. The results obtained on analysis of Low's Champion green beans at various stages during the preparation of these beans for freezing are given (Table 1).

Subsequent studies in which the quantity of acetone used was varied from 50 to 500 c.c. per 25-gram sample indicated a tendency towards preservation of the carotene as the volume of solvent was increased. Other studies indicated that trituration of the samples in sand and acetone followed by refluxing in acetone for approximately 15 minutes preserved the carotene content without loss. This obser-

[1] Approved by the director of the New York State Agricultural Experiment Station as Journal Paper No. 336, July 19, 1939.

[2] This study was conducted under a fellowship donated by the Birds Eye Laboratories of Hoboken, New Jersey.

vation was made on both green beans and Swiss chard. The same carotene content was obtained by heating the samples in acetone as by allowing the blanched (scalded) samples to stand in acetone for 48 hours. Also, extracting freshly ground samples of the same vegetables repeatedly in the cold until free from color gave the same results. These data seem to indicate that there is a great loss of carotene owing to enzyme action during the 48-hour extraction of the pigments from green beans with acetone. Inactivation of the enzyme of the beans by heating in acetone eliminated this difficulty.

Using this procedure, i.e., extraction of the pigments by heating in acetone, samples of green beans obtained toward the end of the growing season were analyzed with the results shown (Table 2).

TABLE 1

Apparent Carotene Content of Green Beans at Various Stages During Preparation for Freezing

Sample	Total solids	Micrograms carotene per gram (fresh basis)
	pct.	
Field	5.99	0.55
After frenching	5.65	0.60
Before scalding	5.69	0.60
After scalding [2 min. at 100°C.(212°F.)]	5.70	3.20
Before freezing	5.93	3.20

This modification of the Russell, Taylor, and Chichester method also possesses other advantages, since heating of the samples in acetone not only permits rapid pigment extraction without noticeable loss of carotene but also facilitates filtration and decreases the tendency towards formation of emulsion during subsequent analysis.

Since it seemed probable that the high results obtained in the determination of carotene in yellow sweet corn by the Russell, Taylor, and Chichester method were caused by the presence of other yellow pigments in the extract, means of eliminating these non-carotenoids were studied.

At this point attention was called to the use of diacetone by Clausen and McCoord (1936) while working on solvent distribution ratios of carotenoid pigments obtained from blood plasma. Later, Hegsted, Porter, and Peterson (1939) employed this solvent for removing non-carotene pigments from corn-silage extracts, abnormally high carotene values having been noted in A.I.V. silages by these workers as well as by Hayden *et al.* (1937) and Peterson *et al.* (1935, 1937).

Quackenbush, Steenbock, and Peterson (1938) found that the carotene fraction obtained from A.I.V. silages contained at least three pigments other than carotene, and that these pigments were inactive biologically and accounted for as much as 40 per cent of the total carotene.

In a new method for determining carotene in silages, Hegsted, Porter, and Peterson (1939) have eliminated the use of 90 per cent methyl and 85 per cent ethyl alcohol and in their place have substituted a solution consisting of 100 volumes of diacetone to six volumes of water, for extracting non-carotene pigments from their solution in petroleum ether. We have found this same procedure applicable when determining carotene in fresh and frozen vegetables.

Preliminary experiments indicated no losses of carotene when

TABLE 2

Carotene Content of Green Beans as Determined by Modified Method

Sample	Total solids	Micrograms carotene per gram (fresh basis)—repeated cold extraction without 48-hour standing	Hot extraction
	pct.		
Immediately after picking	8.26	2.2	2.2
After frenching	7.41	2.1	2.4
After scalding [2 min. at 100°C.(212°F.)]	8.20	2.3	2.1
Before freezing	7.07	2.0	2.0

Golden Cross Bantam corn was heated in acetone during the initial extraction; further, it was shown that considerable amounts of pigment could be removed from the impure petroleum ether solution of carotene by means of the diacetone solvent.

In September, 1938 samples of Golden Cross Bantam corn were obtained for carotene determinations during commercial processing for freezing and storage at the Mount Morris plant of the Snider Packing Corporation, New York. Except for the initial hot-acetone extraction and the use of diacetone, the analyses were conducted essentially as given by the Russell, Taylor, and Chichester (1935) procedure.

After determining the concentration of carotene, the petroleum ether solution was subjected to extraction using five 25-c.c. portions of the diacetone solution; (Hegsted, *et al.* used four 10-c.c. portions in the case of silage). Since further extraction indicated no more loss of color, the petroleum ether was washed with four 50-c.c. portions of distilled water, dried with anhydrous sodium sulfate, and after adjusting to the original 50-c.c. volume, colorimetric readings

were again taken. The average values obtained indicated pigment loss of from 50 to 60 per cent.

No significant differences in carotene content were found between the fresh sample and those obtained during processing for freezing and storage.

PROCEDURE RECOMMENDED

A representative sample of the vegetable is finely cut by means of scissors or knife and the desired quantity weighed as quickly as possible. When preparing the frozen sample it is necessary to precool all apparatus to a temperature well below freezing before use in order to avoid thawing during manipulation. An ordinary meat grinder is satisfactory for comminuting the frozen sample.

The quantity of sample used depends upon the carotene content and varies considerably for different vegetables, e.g., amounting to 25 grams for green beans, 50 grams for corn, 2.5 grams for spinach, 10 grams for broccoli, and 25 grams for asparagus when a final volume of 50 c.c. is used for colorimetric readings.

After being weighed the sample is triturated with sand and 50 c.c. of acetone until in a rather fine state of division; then it is transferred to a 500-c.c. flask and any residual material rinsed from the mortar into the flask, using 100 c.c. of acetone. The flasks are then placed on a suitable bath and heated to boiling for about five minutes.

The acetone solution of the prepared sample is filtered, using a Büchner funnel, and the residue in the flask and funnel is further triturated with two 25-c.c. portions of acetone, the latter being decanted and filtered each time. The residue is then triturated in four successive 25-c.c. portions of petroleum ether, the petroleum ether being decanted and filtered each time. The combined acetone-petroleum ether filtrate is transferred to a liter separatory funnel and 50 c.c. of distilled water is added. (An eight-unit, hand-operated extractor is used in this laboratory.) After complete separation occurs the lower layer is withdrawn and the petroleum ether solution is repeatedly extracted using three 50-c.c. portions of distilled water. If any stubborn emulsions form they can easily be broken by the addition of five to 10 c.c. of saturated sodium sulfate solution. Xanthophyll is next removed by extraction with four successive 25-c.c. portions of 89-per cent methyl alcohol, followed by extraction with four 25-c.c. portions of 25-per cent KOH-methyl alcohol for removal of chlorophyll. After removal of excess KOH by extracting with four to five 50-c.c. portions of distilled water the petroleum ether solution of carotene is extracted using four successive 25-c.c. portions of diacetone solution (100 volumes of diacetone to six volumes of water). The final petroleum ether solution is washed, using

four 50-c.c. portions of distilled water, and after drying with anhydrous sodium sulfate and adjusting to 50-c.c. volume, colorimetric readings are taken.

There is no particular advantage in using both 89-per cent methyl alcohol and diacetone solution as the non-carotene pigments soluble in the alcohol are also soluble in the diacetone solution. In the case of yellow sweet corn, however, a large portion of the yellow pigments which are not removed by the use of 89-per cent methyl alcohol are removed by using the diacetone solution. Whether or not similar pigments may be found in other vegetables remains a subject for future study.

For determining the concentration of carotene in the final petroleum ether solutions, two different photoelectric colorimeters were used at different times. One was equipped with Corning filters No. 428 and 585, the other with Wratten filter C5, No. 47. Both instruments were calibrated against standard solutions of pure beta carotene.[3] Readings taken from both instruments on a beta carotene standard gave concordant results.

In order to evaluate the results obtained by the chemical analyses for carotene the U. S. Pharmacopoeia XI method for vitamin A assay was employed. Quantities of the vegetables stored at —17.8 and —40°C.(0 and —40°F.) were fed at two levels, the first containing the equivalent of .6, the second the equivalent of 1.2 micrograms of carotene. It is necessary to assume that the carotene obtained by chemical analysis is all beta carotene.

For reference purposes groups of animals were maintained which received the following supplements: Groups A and B received one unit; Group C, two units; and Group D, three units of reference cod liver oil per rat per day for the 28-day curative period. Groups E and F received .6; Group G, 1.2; and Group H, 1.8 micrograms of beta carotene per rat per day for the 28-day curative period. The results of these bioassays are presented (Table 3).

A diacetone-solution extract of the petroleum ether solution of pigments obtained from 250 grams of corn, when transferred to Wesson oil and fed to a group of eight animals for the 28-day curative period, indicated that the diacetone-solution-soluble pigments did not possess any biological activity.

DISCUSSION

The present work seems to indicate that there is no loss of carotene in blanched vegetables when stored at —40 or 0°F. for at least a few months. This observation is in accord with DeFelice and Fel-

[3] S. M. A. Corp., Cleveland, Ohio.

TABLE 3

Results of Bioassays of Green Bean and Golden Cross Corn Samples

Material fed	Storage temperature	Total solids	Carotene content in gammas per gram by analysis	Quantity fed daily	Carotene equivalent fed daily in gammas	Number animals started	Number animals finished	Average gain per week	Carotene content in gammas per gram by bioassay
	°F.	*pct.*		*gm.*				*gm.*	
Beans	0	7.45	2.1	0.29	0.6	9	8	14.1	4.1
Beans	0	7.45	2.1	0.58	1.2	8	8	15.7	3.1
Beans	—40	7.12	2.2	0.27	0.6	8	8	13.1	3.7
Beans	—40	7.12	2.2	0.54	1.2	8	8	14.5	2.4
Corn	0	27.57	0.9	0.66	0.6	8	7	7.2	0.6
Corn	0	27.57	0.9	1.32	1.2	8	7	12.6	0.7
Corn	—40	26.66	1.1	0.55	0.6	8	8	10.3	1.2
Corn	—40	26.66	1.1	1.10	1.2	8	7	12.1	0.8
CONTROL GROUPS									
Reference oil (1935)			Group A	1 unit		8	8	12.6	.....
Reference oil (1937)			Group B	1 unit		9	9	12.6	.....
Reference oil (1937)			Group C	2 units		9	9	17.2	.....
Reference oil (1937)			Group D	3 units		9	9	20.7	.....
Beta carotene (1)			Group E	1 unit (0.6 gamma)		9	8	10.0	.....
Beta carotene (2)			Group F	1 unit (0.6 gamma)		8	8	8.2	.....
Beta carotene			Group G	2 units (1.2 gamma)		9	8	14.0	.....
Beta carotene			Group H	3 units (1.8 gamma)		9	8	15.6	.....
Negative control (1)			.....	.....		8	5	—2.2	.....
Negative control (2)			.....	.....		8	5	—3.7	.....

lers (1938) and Fitzgerald and Fellers (1938). The former found no loss in spinach following storage at 0°F. for three months, the same observation having been made by the latter workers in a presentation of the carotene content of 27 different fresh and frozen vegetables.

In some cases the biological assay gave higher results than were to be expected on the basis of the carotene content. This observation has been made by Russell, Taylor, and Chichester (1932) and by DeFelice and Fellers (1938). We find, however, when a comparison is made between the average gain in weight made by the 32 animals which received the single-unit level of the different vegetables and the average gain in weight made by the animals which received .6 microgram or one unit of beta carotene, the results are in good agreement. The same is also true of the two-unit levels. In the former case the average gain made per week by all animals receiving the one-unit level of vegetables amounted to 11 grams as compared with a nine-gram per week average for the reference animals. Statistically, the difference is not significant; in the second case the average gain made per week by all animals receiving the two-unit level of the different vegetables was 14 grams, the same as the reference groups.

SUMMARY

Certain improvements in the Russell, Taylor, and Chichester (1935) method for determining carotene are presented.

A large percentage of the pigments found in Golden Cross Bantam corn, heretofore determined as carotene, is in reality not carotene.

On the basis of the results obtained by the chemical analysis for carotene, frosted green beans and yellow sweet corn were biologically assayed after having been placed in storage at —17.8 and —40°C. (0 and —40°F.) for a period of approximately three to four months.

Better growth response was obtained when increasing levels of reference cod liver oil were fed than when correspondingly increasing levels of pure beta carotene were fed.

The variation in average growth response between those groups fed quantities of vegetables equivalent to .6 microgram of carotene per day (one Int. Unit), was much greater than the variation between the groups which received amounts equivalent to 1.2 micrograms of carotene (two Int. Units).

No appreciable losses of carotene occurred during the storage of the vegetables at either 0 or —40°F.

Carotene analyses as conducted by the modified method are a good index of the vitamin A potency of frozen vegetables.

ACKNOWLEDGMENT

Preliminary studies on the carotene content of sweet corn were conducted by Clinton R. Stimson and reported in a thesis (1939) presented to the faculty of the graduate school for the Degree of Doctor of Philosophy. In his work Stimson found that the Peterson, Hughes, and Freeman (1937) modification of the Guilbert (1934) alcoholic-potash digestion procedure was unsatisfactory for the determination of carotene in corn since large and variable amounts of colored materials were formed in this process which were extracted by the final petroleum ether solutions and measured colorimetrically as carotene. The Russell, Taylor, and Chichester (1935) method of acetone extraction of the pigments proved to give uniform results, which were later found by the biological assay to be much higher than would be expected on the basis of established ratios. The possible explanations for this are given in his thesis and in the present paper. Stimson also observed an apparent increase in the carotene content of the frozen samples of corn over the amount contained in the freshly obtained samples.

From the data collected by Stimson it would appear that the biological potency of sweet corn is much less than one would be led to believe it to be from the abundance of yellow pigments. No loss of carotene seemed to occur in the husking, washing, blanching, cutting, cleaning, packaging, other processes incidental to the preparation for freezing, and in the freezing itself. Losses of carotene seemed to occur after seven months' storage even at as low a temperature as —40°C., but these findings need further confirmation.

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INFLUENCE OF VARIETY AND TREATMENT ON PHYTIN CONTENT OF WHEAT

SILAS M. YOUNG AND J. E. GREAVES
Department of Biochemistry and Bacteriology, Utah Agricultural Experiment Station, Logan, Utah

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The total phosphorus of wheat varies with season, irrigation water applied, manurial treatment, and variety. The question consequently arises: Is it the organic or inorganic phosphorus content which varies? Inasmuch as there is considerable evidence that phytin phosphorus is unavailable to some animals it is important to learn if the phytin content of wheat also varies.

The methods of determining the phytin content of grains are all based on the original Heubner and Stadler (1914) method. They extracted the phytin by soaking finely ground wheat with 1.2 per cent hydrochloric acid. The extract was diluted with an equal volume of distilled water and .03 per cent of ammonium thiocyanate was added as an indicator. This was titrated with a .6 per cent hydrochloric acid containing a known quantity, .05 to 1.2 per cent ferric chloride. Titration was complete when the liquid assumed a faint red color which remained permanent five minutes. Each milligram of iron is equivalent to 1.19 milligrams of phosphorus as phytin.

Rather (1917) extracted the phytin from the grain with 1.2 per cent hydrochloric acid for three hours. Averill and King (1926) found that this concentration of acid did not completely inhibit the action of phytase, hence better results were obtained with two per cent hydrochloric acid. These workers used eight grams of finely ground grain in 200 c.c. of acid. Fifty c.c. of the filtered extract were titrated using 10 c.c. of .3 per cent ammonium thiocyanate as an indicator.

The end point is rather indefinite, owing to the formation of a slowly settling colloidal precipitate of ferric phytate. Andrews and Bailey (1932) suggested that this be overcome by titrating short of the end point, filtering, and finishing the titration on the filtrate. Harris and Mosher (1934) prefer to titrate past the end point, filter, and match colors with a blank. Knowles and Watkin (1932) thought that sodium salicylate gave a sharper end point than ammonium thiocyanate. Young (1936) used a definite amount of ferric chloride, precipitating the ferric phytate at 100°C.(212°F.), and determined the excess iron by the thiocyanate method.

EXPERIMENTAL PROCEDURE

The above-outlined procedures were checked and the following conclusions reached: (1) the method of comparing the color of the sample with that of a blank was found to be difficult, as the blank was pink whereas the sample was brown; (2) little difference was noted in the sharpness of end point for either ammonium thiocyanate or sodium salicylate as the indicator; (3) the stronger solution of ferric chloride gave a sharper end point than did the weaker. As a result of this preliminary work the method selected was as follows: (1) eight grams of finely ground wheat were soaked in 200 c.c. of two per cent hydrochloric acid for three hours and then filtered through two layers of hardened filter paper; (2) 50 c.c. of the filtrate was placed in a 400-c.c. beaker, and 10 c.c. of .3 per cent ammonium thiocyanate solution added as an indicator; (3) 107 c.c. of distilled water was then added to give it the proper acidity; (4) this was titrated with a standard ferric chloride solution which contained about .00195 gram of iron per cubic centimeter, until a brownish-yellow color persisted for five minutes. From the number of cubic centimeters of ferric chloride used the iron equivalent was obtained, and from this the amount of phytin phosphorus was found by multiplying by the factor 1.19. This was converted into phytin by multiplying by the factor 3.55.

Thirty-two samples of wheat grown at the Nephi dry-farm substation under known conditions[1] were tested for phytin content. They were divided into two general groups. The first group consisted of 21 samples of different varieties, all grown, harvested, threshed, stored, and handled alike. The second group contained 11 samples of the same variety (Kanred) grown under different manurial treatments.

Two eight-gram samples of each variety of wheat were extracted. From each extraction three aliquots of 50 c.c. were taken for titration, making a total of six titrations on each sample. The average of the six readings was taken as the value for that sample.

DISCUSSION OF RESULTS

The total phosphorus determinations have been reported on these wheats by Greaves and Hirst (1929a). From their results the total phosphorus, the percentages existing in the form of phytin, and the non-phytin phosphorus were calculated. The percentages of phytin phosphorus in the sample, the percentages of total phosphorus, and

[1] The varieties of wheat analyzed were furnished by A. F. Bracken and were grown at the Nephi dry-farm substation.

the percentages of the total phosphorus that is phytin phosphorus for the various varieties of wheat are given (Table 1).

The lowest percentage of phytin phosphorus found was .152 per cent in the sample of Newturk wheat, as compared with a high of .328 per cent for Dicklow (more than double that of Newturk). Kofod wheat carried the lowest percentage of the total phosphorus

TABLE 1

Phytin Phosphorus, Total Phosphorus, and Percentage of Total That Is Phytin Phosphorus in 21 Varieties of Wheat

Variety	Phytin phosphorus	Total phosphorus	Per cent of total that is phytin phosphorus
	pct.	*pct.*	
Newturk	.152	.247	61.5
Kofod	.158	.302	52.3
Regal	.164	.289	56.7
Black Hull	.167	.268	62.3
Kanred and Hussar cross	.167	.238	70.0
Turkey and Hussar cross	.181	.235	77.0
Tenmarq	.186	.284	65.5
Turkey	.186	.258	72.0
Sevier 34-1	.186	.280	66.3
Sevier 59-2	.190	.272	70.0
Ora	.195	.237	82.0
Montana	.195	.282	69.2
Kharkof	.200	.276	72.5
Goldcoin	.204	.233	87.5
Relief	.204	.244	83.5
Kanred	.213	.279	76.3
Alton	.216	.271	80.0
Baart	.222	.287	77.2
Federation	.272	.288	94.3
Hard Federation and Dicklow cross	.322	.378	85.0
Dicklow	.328	.350	93.5

that was in the form of phytin—52.3 per cent; Federation contained the highest 94.3 per cent. It is evident that there is a wide variation in the total phosphorus content of these wheats and that the quantity which is in the form of phytin varies widely, but the phytin content does not necessarily vary with the total phosphorus content.

The grams of phytin phosphorus, total phosphorus, and grams of non-phytin phosphorus in 1,000 grams of wheat can be readily calculated from Table 1. When this is done Dicklow is found to carry the smallest quantities of phosphorus in forms other than phytin, .22 gram per 1,000 grams of wheat; and Kofod had the greatest amount, 1.44 grams per 1,000 grams of wheat. Kanred and Baart

have practically the same amount, .66 and .65 gram per 1,000 grams of wheat respectively.

If, as claimed by some workers, phytin phosphorus is unavailable, an animal eating 1,000 grams of Dicklow would receive 3.5 grams of total phosphorus, only .22 gram of which would be available; whereas, if the animal received 1,000 grams of Kofod it would receive 1.44 grams of available phosphorus. Hence, an animal eating Dicklow would receive over twice the phosphorus of one which ate Kofod, but the one eating Kofod would receive nearly seven times as much non-phytin phosphorus. If phytin phosphorus is unavailable, these wheats vary widely in their available phosphorus.

TABLE 2

Phytin Phosphorus, Total Phosphorus, and Percentage of Total Phosphorus for Kanred Wheat Receiving Different Treatment

Treatment for fallow	Phytin phosphorus	Total phosphorus	Per cent of total that is phytin phosphorus
	pct.	*pct.*	
Fall-plowed	.222	.301	73.7
Straw-burned	.223	.278	80.0
High-stubble-plowed	.224	.320	70.0
High-stubble-burned	.246	.285	86.3
Peas-in-pod-plowed	.257	.302	85.0
Wheat-six-inches-high-plowed	.269	.326	82.5
Peas-12-inches-high-plowed	.269	.317	85.0
Peas-six-inches-high-plowed	.269	.291	92.5
Wheat-in-bloom-plowed	.281	.366	76.8
Wheat-in-milk-plowed	.286	.321	89.2
Peas-in-bloom-plowed	.292	.313	93.0

The percentage of phytin phosphorus, percentage of total phosphorus, and percentage of the total phosphorus that is phytin phosphorus for the Kanred wheat grown under different conditions are shown (Table 2).

Large variations in phytin content were found among the second group of wheats also. The sample from the plot which was fall-plowed was low in phytin phosphorus—.222 per cent. The wheat from the plot where peas had been plowed under while in bloom was high—.292 per cent. High-stubble-plowed-under was low in percentage of the total phosphorus that is phytin phosphorus—70 per cent—whereas peas-plowed-under-while-in-bloom was high—93 per cent. Wheat-six-inches-high, peas-12-inches-high, and peas-six-inches-high all give the same percentage of phytin phosphorus. The fall-plowed, straw-burned, and high-stubble-plowed-under treatments

showed no great difference. Two of the samples, peas-in-pod and peas-12-inches-high-plowed-under, gave the same percentages of total phosphorus that was in the form of phytin phosphorus.

If we calculate the grams of phytin phosphorus, grams of total phosphorus, and grams of non-phytin phosphorus in 1,000 grams of Kanred wheat receiving the various treatments we find that 1,000 grams of wheat, grown under the peas-in-bloom-plowed-under treatment was low—.21 gram—and high-stubble-plowed-under was high—.96 gram. Peas-six-inches-high and peas-in-bloom gave very nearly the same weight of non-phytin phosphorus in 1,000 grams of wheat, as did also the straw-burned and the wheat-six-inches-high-plowed-under. Hence it is probable that cultural treatments very materially change the nutritive value of wheat.

Greaves and Hirst (1929b) found that many Utah-grown wheats varied in organic and inorganic phosphorus. They found an average percentage of total phosphorus of .363 per cent, and inorganic phosphorus of .023 per cent, which left an average organic content of .340 per cent of the grain. These wheats carry an average phytin phosphorus of .205 per cent. The highest was .328 per cent, and the lowest was .152 per cent; hence, approximately two-thirds of the organic phosphorus is in the form of phytin. Greaves and Hirst (1929b) found that the average amount that is organic phosphorus is 93.6 per cent of the total phosphorus. The average amount of the total phosphorus that is phytin phosphorus is 74.3 per cent. According to Kolobkova (1937) the organic phosphorus of wheat is in the form of phytin, lecithin, and protein.

Greaves and Nielson (1936) found that Kofod wheat when fed to rats raised the blood phosphorus the least of the varieties listed and that Regal wheat was least effective in the cure of rickets, as noted by the line test of the tibia. There is no correlation between the ability to prevent rickets and the phytin content, as both Kofod and Regal have a low percentage of the total phosphorus as phytin phosphorus, 52.3 and 56.7 per cent, respectively. They are both high in non-phythin phosphorus, 1.44 and 1.25 grams, respectively, per 1,000 grams of wheat. These results indicate, as did the findings of Harris and Bunker (1935), that other factors besides phytin are present to affect the anticalcifying action.

Other workers—Arberg (1922), Averill and King (1926), and Rather (1917)— in general, obtained higher percentages of phytin in wheat and related products than were found in this work. For example, their results showed that Baart wheat contained .327 per cent phytin phosphorus, as compared with .222 per cent found in

these experiments. This difference may be caused by factors such as different soil and water conditions and the rate of growth.

SUMMARY

Twenty-one samples of different wheat varieties and 11 samples of the same variety grown under different fertilization treatments were tested for their phytin content by a modification of the method of Heubner and Stadler (1914), in which the phytin is precipitated out of an acid extract of the grain by titration with ferric chloride in the presence of a suitable indicator.

Some of the wheats were found to carry twice as much phytin phosphorus as others. There were greater differences between varieties than within the same variety under different treatments. The amounts of phytin phosphorus varied from 1.52 to 3.28 grams per 1,000 grams of wheat, or an average of 2.05 grams per 1,000 grams of wheat. The phytin phosphorus varied from 52.3 to 94.3 in percentage of the total phosphorus with an average of 74.3 per cent. The amounts of phosphorus not in the form of phytin varied from .16 to 1.44 grams per 1,000 grams of wheat, or an average of .71 gram per 1,000 grams of wheat.

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A COMPARATIVE STUDY OF THE CHEMICAL COMPOSITION OF FIVE VARIETIES OF SOYBEANS

R. C. BURRELL AND ALVIN C. WOLFE
Ohio State University, Columbus, Ohio

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Undesirable taste and texture of many varieties of soybeans limit their use as human food. Out of a large number of varieties that have been tested by Woodruff and Klaas (1938) for palatability, taste and texture only a few have been found satisfactory. Even with these few acceptable food varieties, certain differences in taste and texture are noticeable.

It seemed possible that the "nut-like" consistency of some varieties and the "mushy" consistency of other varieties might be due to differences in composition of the cell walls of the beans. If a correlation between chemical composition and taste or texture could be confirmed, it should be possible to make use of such information in breeding varieties with more desirable characteristics.

The purpose of this investigation was to make a comparative analysis of several varieties[1] of soybeans that were grown, harvested, and stored under similar conditions and which included the poorest as well as the best edible varieties; and to determine if any correlations exist between edible qualities and chemical composition.

Bansei, Jogun, and Kura were selected as representatives of the better edible varieties and Illini and Peking as representatives of varieties of inferior quality.

METHODS OF ANALYSIS

Official methods of analysis, of the Association of Official Agricultural Chemists (1930), were employed in the determination of moisture, ash, ether extract, protein, and crude fiber. Moisture was determined by distilling 25-gram samples of the soybean meal with toluene and collecting the water in Bidwell-Sterling tubes. Ash was determined by charring two-gram samples in platinum dishes over a low flame and heating in a muffle furnace for 14 hours. To obtain the percentage of crude fat or ether extract, two-gram, air-dried samples were extracted 16 hours with anhydrous ether. The extract was dried in an oven at 100°C.(212°F.) for one hour and weighed.

[1] The soybeans were supplied by Dr. J. B. Park of the Department of Agronomy, to whom the authors are also indebted for helpful suggestions concerning the selection of suitable varieties.

Some years ago Street and Bailey (1915) analyzed a Hollybrook variety of soybean for the carbohydrate constituents. Their general procedure with certain modifications was adopted in this investigation for the analysis of the carbohydrate portion. The finely ground soybean meal, freed from fat with ethyl ether, was extracted successively with boiling, 95-per cent, ethyl alcohol; cold water, taka-diastase preparation; and one per cent of hydrochloric acid. The kind and quantity of the chief carbohydrates present in these extracts and residue were then determined. Total galactans were determined in separate samples of the meal, using the tentative procedure given in "Official Methods" (1930). Starch determinations were also made on separate samples by the calcium chloride-extraction method of Denny (1934).

In the extraction with 95-per cent alcohol, excessive bumping was avoided by placing samples of the fat-free meal in large extraction thimbles and immersing these thimbles in boiling alcohol for 16 hours. The thimbles were then placed in Soxhlet extractors and extracted for eight hours with fresh 95-per cent alcohol to wash out the remaining solutes. The two extracts were combined, the alcohol was distilled off, and the residue was dissolved in water. The water solution was clarified with neutral lead acetate and the excess lead was removed with anhydrous sodium sulfate. The clear, straw-colored solution was diluted to 250 c.c. and aliquots were used for the determination of reducing sugars, sucrose, and raffinose by "Official Methods" (1930).

The residue from the alcohol extraction was dried, treated with cold water for 24 hours, filtered, and washed with cold water. The filtrate and washings were concentrated at 70°C.(158°F.) with sodium carbonate added to neutralize the acidity. The solution was then made up to 500 c.c. and 100-c.c. aliquots were clarified with neutral lead acetate and de-leaded. Reducing activity was determined by use of Fehling's solution. Reduction after hydrolysis was similarly determined with 50-c.c. aliquots.

Starch was determined in the residue from the cold-water extract by hydrolyzing with taka-diastase according to the standard procedure. Using samples of the ether-extracted meal, starch was also determined by the method of Denny (1934).

A portion of the residue from the starch determination was refluxed for one hour with 200 c.c. of boiling, one-per cent, hydrochloric acid and then filtered. From the reducing action of the neutralized filtrate on Fehling's solution, the percentage of reducing substances originating from the mild hydrolysis of cell-wall constituents was calculated.

Samples of the residue from the starch determination were examined for their pentosan and galactan content by "Official Methods" (1930).

The average quantities of the above-mentioned components which were found present in the five varieties of soybeans are shown (Table 1).

ASH ANALYSIS

The ash was analyzed for calcium, magnesium, and phosphorus by a method used by Morris, Nelson, and Palmer (1931) for the quantitative determination of these elements in cattle excreta and feedstuffs.

TABLE 1

Average Percentages of Certain Components in Moisture-Free, Mature Soybeans

Component	Jogun	Bansei	Kura	Illini	Peking
Ash	5.17	5.01	5.13	4.44	5.13
Fat (ether extract)	19.03	20.17	17.64	20.02	16.69
Protein	41.87	40.90	46.08	43.67	39.30
Crude fiber	4.43	4.44	4.66	5.33	6.41
Reducing sugars (dextrose)	0.135	0.116	0.053	0.110	0.229
Sucrose	4.00	4.75	6.39	5.21	5.65
Raffinose	0.86	0.95	1.12	1.04	0.95
Cold-water extract (dextrins)	3.25	5.53	4.02	4.30	2.39
Starch	None	0.16	None	None	None
One per cent acid hydrolysis (as dextrose)	4.45	4.35	3.31	3.27	3.87
Pentosan	3.06	3.21	2.51	3.24	3.09
Galactans of residue	0.214	0.405	0.261	0.175	0.086
Total "galactans"	2.08	1.77	1.30	1.70	1.15

In this method the calcium was precipitated as the oxalate by a modification of the Kramer and Howland (1926) procedure, while a washing and draining method was adopted from Clark and Collip's (1925) blood-calcium procedure. The calcium oxalate was dissolved in hot, normal, sulfuric acid and titrated with .01 N potassium permanganate solution.

The magnesium was precipitated as the phosphate according to the Kramer and Howland method and the precipitated phosphate determined colorimetrically by the method of Fiske and Subbarow (1925).

Phosphorus was determined colorimetrically by reducing the ammonium phospho-molybdate with amino-naphthol-sulfonic acid and comparing the deep blue color with a standard made up from pure potassium di-hydrogen phosphate.

The average percentages of calcium, magnesium, and phosphorus in the moisture-free, mature soybeans are shown (Table 2).

DISCUSSION

The sum of the percentages of sucrose and raffinose determined from polarization readings was in every instance higher than the percentage of sucrose and raffinose found after inversion with hydrochloric acid and determination of reduction with Fehling's solution. This disagreement may be due to the presence of some undetermined substance with high rotatory power, such as stachyose, which has been reported as present in soybeans.

The differences in the total "galactans" as determined for the fat-free meal and the galactans of the residue, indicate the presence of considerable quantities of soluble, mucic acid-yielding substances.

TABLE 2

Average Percentages of Calcium, Magnesium, and Phosphorus in Moisture-Free, Mature Soybeans

	Jogun	Bansei	Kura	Illini	Peking
Calcium	.227	.194	.292	.272	.286
Magnesium	.238	.259	.189	.235	.253
Phosphorus	.247	.251	.253	.224	.276

No reducing substances were found present in the cold-water extract before hydrolysis.

Starch values obtained by the two different methods did not agree. According to the calcium chloride extraction procedure, Bansei was the only variety which contained starch. With the standard method values up to .646 per cent were obtained after suitable corrections for the blank determination had been made. It is believed that the calcium chloride method gives the more reliable results.

The investigation is being extended to a study of relations between quality—Woodruff and Klaas (1938) and Munger (1937)— and the composition of varieties edible in the green stage with particular reference to changes in composition at different periods of development. It is, of course, recognized by Munger (1937) that other factors besides carbohydrates are responsible for the taste and texture of soybeans. Changes in the protein and lipide fractions are also being followed. From the acetone extract of field varieties material has been obtained which contains a bitter substance. This has not yet been completely purified, but the impure preparation gives several reactions characteristic of pyrogallic acid. The steam distillate contains a very small quantity of a substance which possesses the dis-

tinctive "beany" taste. It does not respond to tests for aldehyde, ketone, or unsaturated groupings. On standing for some time, the odor of this watery distillate changes and it becomes progressively more acid, indicating the possibility of an ester.

The three edible varieties, Jogun, Bansei, and Kura, contain less crude fiber than do the two field varieties, Illini and Peking. No other important difference was found between edible and field varieties.

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CLOSTRIDIUM PASTEURIANUM ASSOCIATED WITH SPOILAGE OF AN ACID CANNED FRUIT[1]

C. H. SPIEGELBERG
Pineapple Experiment Station, Honolulu, Territory of Hawaii

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In a study of types of spoilage in an acid canned fruit (*Ananas comosus* Merr.) the writer found both spore-forming and non-spore-forming bacteria. The spore-formers proved to be obligate anaerobes of the genus *Clostridium*, Spiegelberg (1936), and were of two general types which appeared to be smooth (S type) and rough (R type). Pure-culture inoculations with the two types into commercially canned pineapple gave swells like those encountered in commercial packs. This paper gives a description of the clostridia and a comparison of them with previously described species of butyric anaerobes of the soil.

SOURCE OF THE CULTURES

All of the cultures investigated were obtained from bursting cans occurring in packs of canned pineapple during 1930 to 1934. Pure cultures were readily obtained in nutrient one-per cent dextrose agar by use of Spray (1930) culture dishes or by the pyrogallic-acid tube method of Hall (1937). Aerobic spore-formers, mostly of the *Bacillus subtilis* type, which were often encountered in both normal and swelled cans, were readily eliminated by the above isolation methods but persisted in plates incubated in oat jars. Stock cultures were maintained in the ice box on nutrient one-per cent dextrose agar slants and were transferred at intervals of six months to a year. Approximately 90 cultures were isolated and examined for colony characteristics, growth in nutrient dextrose (one per cent) broth, pineapple juice, litmus milk, gelatin, and brain mash. Of these, 34 cultures were selected for more detailed study as reported in this paper.

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS

Morphologic characters seem to be of little value in separating species of butyric anaerobes, according to Bredemann (1909); Bushnell (1922); McCoy, Fred, Peterson, and Hasting (1930); and Sjolander and McCoy (1937). However, the smooth (S) and rough (R) types differed in several characters.

[1] Published with the approval of the director as Technical Paper No. 118 of the Pineapple Experiment Station, University of Hawaii.

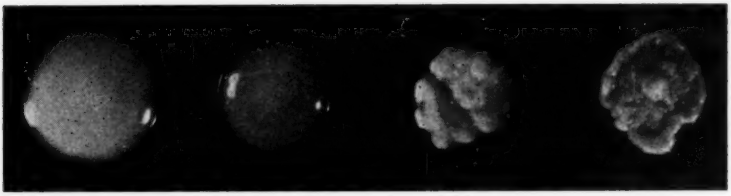

FIG. 1. Smooth and rough colonies from beef extract (one per cent dextrose) agar plates (Spray dishes). Incubated five days at 37°C. (98.6°F.). Enlarged approximately 7X.

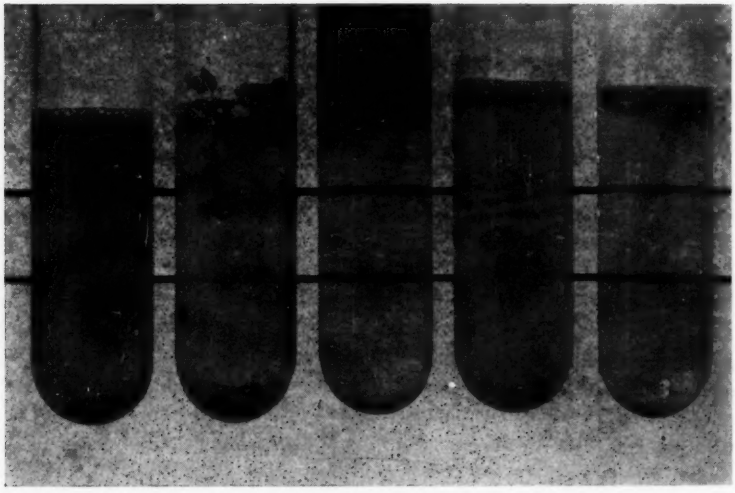

FIG. 2. Growth of Cultures 31(S) and 57(R) in pineapple juice and beef extract (one per cent dextrose) broth. Reading from left to right: the first two tubes are smooth and rough types in pineapple juice of pH 5; the third tube is a check; and the last two tubes are smooth and rough types in beef extract broth. Note differences in turbidity of S and R types, especially in beef extract broth, and the bacterial clumps produced by the R type in both pineapple juice and beef extract broth.

The cultures are gram-positive, catalase negative. Vegetative cells and young clostridia of the S type are actively motile, flagella being peritrichiate, but the R type is usually sluggishly motile. Young clostridia show the characteristic granulose reaction when stained with iodine. Spores are formed readily on agar, are terminal or subterminal, oval to round, and are retained within the sporangia, which may be elongated, club-shaped, or spindle-shaped.

On nutrient dextrose agar surface colonies of the S type are slightly raised, milky white to cream colored, circular, and smooth with entire margin. The R type colonies are creamy yellow, raised, irregular in outline, and the surface is crateriform or papillate giving "piled up" colonies (Plate 1, Fig. 1). In nutrient dextrose broth the S type produces a heavy even clouding which settles out slowly, while the R type clumps at the bottom of the tube leaving the liquid almost clear. Gas production may raise the clumps to the surface where they hang in irregular strands (Plate 1, Fig. 2).

PHYSIOLOGICAL CHARACTERISTICS

In all of the following tests, duplicate tubes were inoculated with two to four drops of active culture and incubation was at 37°C. (98.6°F.). Plate cultures with clay tops were incubated in oat jars. Only those tests which have been found useful in the classification of butyric anaerobes were included, McCoy, *et al.* (1930).

Gelatin: None of the cultures liquefied plain gelatin, gelatin plus one per cent dextrose, or gelatin plus one per cent sucrose when incubated at 37°C. under vaseline seal.

Litmus Milk: Little change was produced in litmus milk. A slight amount of acid and gas was produced but no curd was formed after two weeks' incubation. Reduction of litmus is sometimes noted during early stages of growth.

Hydrogen Sulfide: Good growth was obtained in Hall's (1937) deep brain medium but there was no liquefaction or blackening either with or without iron. A tryptone (.5 per cent) dextrose (.5 per cent) broth with reduced iron and sodium sulfite (.25 per cent) turned nearly black as did lead acetate papers suspended in the tubes. A test to determine the tolerance of sulfite was suggested by the paper of Partansky and Henry (1935). Sodium sulfite in concentrations of .25, .50, 1, 2, and 4 per cent was added to the tryptone-dextrose-iron medium. Good growth and blackening occurred only in the media containing .25 and .50 per cent sodium sulfite. No direct comparison can be made of these results with those obtained by Partansky and Henry, since those authors used only sulfite waste liquor; however, the results do not indicate high tolerance of sulfite. *Clostridium pasteurianum* (Winogradsky strain) and *Clostridium acetobutylicum*

were also included in this test. The former gave results identical with those produced by the pineapple cultures, but *Cl. acetobutylicum* gave positive results only in .25 per cent sodium sulfite. Even in this concentration, there was no darkening of the medium and only a slight blackening of the lead acetate paper.

Nitrate Reduction: No nitrite was produced in beef extract broth containing .1 per cent each of dextrose and potassium nitrate.

TABLE 1

Summary of Fermentation Reactions

Test substance	Number of cultures tested	Gas [1]		Acid [2]		Titration of media	Fermentation of compound
		Min.	Max.	Min.	Max.		
Pentose:							
Arabinose	8	Trace	Trace	0.4	0.6	0.6	0
Hexoses:							
Glucose	34	23	38	5.4	6.9	0.6	+
Galactose	8	16	24	4.0	5.4	0.4	+
Disaccharides:							
Sucrose	34	22	32	5.4	6.4	0.4	+
Lactose [3]	8	Trace	Trace	0.4	0.6	0.4	0
Polysaccharides:							
Starch [4]	34	....	....	....	....	....	0
Dextrin	8	Trace	1	0.8	1.1	0.7	0
Glycogen	8	Trace	Trace	0.6	1.1	0.6	0
Cellulose	8	Trace	Trace	0.3	0.8	0.7	0
Pectin	34	Trace	1	2.0	2.8	2.1	0
Glucosides:							
α methyl glucoside	8	18	33	4.2	6.1	0.4	+
Salicin	8	Trace	Trace	0.5	0.9	0.4	0
Amygdalin	8	Trace	Trace	0.4	0.8	0.4	0
Esculin	8	Trace	Trace	2.0	3.7	2.4	0
Alcohols:							
Glycerol	34	8	25	2.4	4.0	0.4	+
Mannitol	34	12	32	2.6	5.0	0.5	+
Erythritol	8	Trace	Trace	0.3	0.6	0.4	0
Organic acid:							
Calcium lactate	34	Trace	Trace	0.2	0.5	0.6	0
Control [5]	34	Trace	Trace	0.2	0.4	0.4	0

[1] Rise (cm.) of vaseline plugs in tubes (diameter approximately 15 mm.) containing 10 c.c. of medium. [2] C.c. 0.1 N NaOH to neutralize 10 c.c. of culture. Minimum and maximum amounts of acid and gas are for lowest and highest readings obtained with all cultures tested. [3] Reaction in litmus milk also indicates failure to ferment lactose. [4] Tested on starch-agar plates and corn-mash tubes. [5] Same inoculum in basal medium without added test substance.

Corn Mash: Corn mash cultures with and without liver, McClung and McCoy (1934), showed only traces of gas and no liquefaction of the medium, corresponding to Group 1a, *Cl. pasteurianum* type of McCoy, *et al.* (1930).

Diastatic Action: There was no evidence of hydrolysis of starch on plate test.

Fermentation: Fermentation of carbohydrates, higher alcohols, glucosides, and calcium lactate was determined in a medium consisting of .5 per cent tryptone broth and one per cent of the test substance, the two being sterilized separately in double strength. Filter-paper strips were used for the cellulose series. The positive cases (Table 1) are indicated by gas and acid production exceeding that in inoculated tubes containing basal medium and no added test substance, and are compared with uninoculated controls. Positive fermentations were obtained with glucose, galactose, sucrose, α methyl glucoside, glycerol, and mannitol. There was no utilization of arabinose, lactose, dextrin, glycogen, cellulose, pectin, aesculin, salicin, amygdalin, erythritol, or calcium lactate.

PRODUCTS OF FERMENTATION

In a comprehensive study of butyric-acid-forming anaerobes McCoy, *et al.* (1930) defined the butyric anaerobes of fermentation as "a subgroup of clostridia, nonpathogenic, yielding larger amounts of butyric acid or characteristic neutral products in addition, and giving the granulose reaction." Two groups were considered—Group 1, "true butyric" anaerobes, which produce acid end products, chiefly butyric and acetic acids; and Group 2, the "butyl organisms," which produce butyric and acetic acids as intermediate products and alcohols or alcohols and acetone as end products. Group 1 was further subdivided into non-starch-fermenting clostridia (*Cl. pasteurianum* type) and starch-fermenting clostridia or plectridia [*Bacillus (Cl.) saccharobutyricus* type]. Sjolander and McCoy (1937) and Langlykke, Peterson, and McCoy (1935) also studied the fermentation products of a number of butyric anaerobes and found considerable variation in proportions of the various products.

In the present study utilization of glucose was determined in four basal media as follows:

1. Double-strength[2] yeast water, adjusted to pH 7.0.
2. .5 per cent tryptone.
   .1 per cent asparagine.
   .1 per cent $(NH_4)_2HPO_4$.
3. 2 per cent malt sprouts.
4. .05 per cent $MgSO_4$.
   .20 per cent $(NH_4)_2SO_4$.
   .30 per cent $(NH_4)_2HPO_4$.
   .05 per cent NH_4Cl.
   .50 per cent tryptone.
   Distilled water.

[2] Two hundred grams of starch-free yeast extracted in 1,000 c.c. of water.

Glucose (sterilized separately) and calcium carbonate were added as shown (Table 2). Large tubes containing 40 c.c. of medium were inoculated with Cultures 31 (S) and 57 (R) and incubated for five days at 37°C.(98.6°F.). Residual sugar was determined by the method of Stiles, Peterson, and Fred (1926). The amount of glucose utilized was similar for both types of organisms and depended more upon the amount of calcium carbonate present than upon nitrogen source.

The nature of the fermentation products from glucose was determined in double-strength yeast water. Analytical methods were the

TABLE 2

Utilization of Glucose[1] in Various Base Media[2] With Indicated Additions of Glucose and Calcium Carbonate

Additions		Culture No. 31 (S)				Culture No. 57 (R)			
		Base media				Base media			
Glucose	$CaCO_3$	1	2	3	4	1	2	3	4
pct.	*pct.*								
2.0	0.0	76	55	28	55	73	71	35	65
	0.1	83	....	38	54	83	68	47	67
	1.0	98	99	97	99	98	99	98	99
3.5	0.0	49	40	25	36	43	45	43	44
	0.1	48	44	32	39	53	50	41	45
	1.0	91	96	98	99	80	99	99	99
5.0	0.0	39	30	16	24	43	35	24	31
	0.1	40	33	25	28	38	33	30	29
	1.0	70	60	62	75	69	72	70	73

[1] All data expressed as per cent of the available glucose. [2] See text for composition of these media.

same as used by Langlykke, *et al.* (1935). In one set of cultures no calcium carbonate was added and solvents were determined at the completion of fermentation. The results (Table 3) show that quantities of solvents produced are negligible and that the main products are acidic. In another test, yeast water with calcium carbonate was inoculated with Culture 31 (S); and after fermentation was complete, volatile acids (Table 4) were determined by the Duclaux method as modified by Virtanen and Pulki (1928). Approximately 50 per cent of the sugar utilized was converted into butyric and acetic acids. The production of large quantities of hydrogen and carbon dioxide probably accounts for most of the balance of the sugar, since other volatile products were almost completely absent (Table 3).

NITROGEN FIXATION

The fixation of atmospheric nitrogen by anaerobic spore-forming bacteria was first described by Winogradsky (1894) for the organism which he called *Cl. pasteurianum*. Winogradsky considered the ability to fix atmospheric nitrogen as peculiar to this organism. McCoy, Higby, and Fred (1928) showed that the ability to fix atmos-

TABLE 3

Production of Solvents From Glucose (2.41 Per Cent) in Double-Strength Yeast Water

Culture	Glucose		Acidity N/10 NaOH in 10 c.c.	Neutral volatile products					
				Butyl alcohol	Ethyl alcohol	Acetone	Isopropyl alcohol	Total	Glucose used
	mg./ 100 c.c.	*pct.*	*c.c.*	*mg./ 100 c.c.*	*mg./ 100 c.c.*	*mg./ 100 c.c.*	*mg./ 100 c.c.*	*mg./ 100 c.c.*	*pct.*
31 (S).....	1355	56.2	8.30	22.0	24.0	10.0	0	56.0	4.1
57 (R).....	1170	48.6	7.30	19.5	23.0	12.0	0	54.5	4.7

pheric nitrogen is not distinguishing for *Cl. pasteurianum* but that other butyric anaerobes have this ability in some degree. "Generally speaking, however, the non-starch fermenting *Cl. pasteurianum* types fix most nitrogen and they fix most consistently in successive experiments."

Sjolander included two strains, 31 (S) and 57 (R), of the pineapple clostridia in a study of nitrogen fixation by several species of butyric anaerobes. Methods employed were those used by McCoy,

TABLE 4

Production of Acids From Glucose (Three Per Cent) in Double-Strength Yeast Water Containing One Per Cent $CaCO_3$

Culture	Glucose		Acid produced			
			Acetic	Butyric	Total	Glucose used
	mg./ 100 c.c.	*pct.*	*mg./ 100 c.c.*	*mg./ 100 c.c.*	*mg./ 100 c.c.*	*pct.*
31 (S).....	2970	99	520	830	1350	45.5

et al. (1928). Six-ounce bottles containing 100 c.c. of Winogradsky's medium plus 1.64-1.80 per cent dextrose were inoculated in triplicate with liver broth cultures and incubated for 24 to 25 days at approximately 28°C.(82.4°F.). Controls consisted of medium inoculated with sterile liver broth and medium inoculated with active culture and killed immediately by addition of sulphuric acid. Both cultures utilized 100 per cent of the added sugar. Culture 31 (S)

fixed 2.60, 2.60, and 2.55 mg. of nitrogen, while Culture 57 (R) fixed 2.00, 2.10, and 2.10 mg. nitrogen for the three bottles (per 100 c.c.).

MISCELLANEOUS TESTS

The isolation of these clostridia from swelled canned pineapple, an acid product with approximately 25 per cent of sugar, led to a study of pH and sugar tolerance and to thermal death-time determinations of the organisms. A detailed report of the results will be given elsewhere, hence only summary statements are presented here.

pH Tolerance: In experimental packs of canned pineapple involving many hundreds of cans, no butyric spoilage developed at pH below approximately pH 4.4 to 4.5, even when a sub-standard process was given. With pH from 4.5 to 5.0 the percentage of swells increased greatly. When commercially sterile cans were inoculated with active cultures, a few cans of pH 4.2 swelled but the percentage of swells increased progressively with increase of pH up to 4.8 to 4.9 at which values all inoculated cans swelled.

In nutrient dextrose (one per cent) broth the cultures failed to develop at approximately pH 4.2 but developed well at pH 5.0, 6.0, and 7.0.

Waksman (1932) states: "The optimum reaction (for *Cl. pasteurianum*) is pH 6.9-7.3, but the organism still develops well at pH 5.7. It can be found in soils even with a pH of 5.0 to 5.2." While our cultures tolerate greater acidity than indicated for *Cl. pasteurianum* by Waksman, Townsend (1938) worked with cultures which grew in pear juice of pH 3.55. Van Beynum and Pette (1935) found that butyric anaerobes were readily isolated from silage of pH 3.0-4.0.

Sugar Tolerance: The pineapple clostridia were compared with a number of other butyric anaerobes, including *Cl. pasteurianum* (No. 5 of McCoy, *et al.* 1930), in tryptone (one per cent) broth containing varying amounts of sucrose or glucose. *Cl. pasteurianum* and the pineapple clostridia produced identical results in all tests, each fermenting sucrose in concentrations ranging from one to approximately 40 per cent and glucose up to about 27 per cent. *Cl. saccharobutyricus* v. Klecki. the next most active culture, fermented in 30 per cent sucrose and 15 per cent glucose. *Cl. butyricum* Prazm., *Cl. butylicum* Beij., *Cl. butylicus* Fitz, and *Cl. beijerinckii* Donker all showed less tolerance to both sucrose and dextrose.

Thermal Death Time: The methods used were similar to those described by Esty (1928). Spores were heated in nutrient broth of pH 5.0 and subcultured in broth of pH 6.5 to 6.7. The number of spores was varied from approximately 14,000 to 50,000,000 per c.c. The smooth strains proved to be somewhat more resistant than the

rough strains but neither could be considered highly resistant, since all failed to resist 100°C.(212°F.) for approximately five minutes. The S type spores, in concentrations of 14,000 and 140,000 per c.c., were killed in 10 minutes at 95°C.(203°F.), but in higher concentrations were not all killed by this treatment. The R type failed to survive 95°C. for 10 minutes in any concentration but did survive 90°C.(194°F.) for 10 minutes.

SEROLOGY

The successful use of serological agglutination technique to establish identity of various species of anaerobic spore-forming bacteria has been reported by McCoy (1934), McCoy and McClung (1935, 1938), Smith (1937), and others. The object of the present study was to determine whether the S and R strains of pineapple organisms were related serologically and also to compare these organisms serologically with two species, *Cl. pasteurianum* Winogradsky and *Cl. beijerinckii* Donker, to which they seemed most closely allied on morphological and physiological grounds.

The methods employed were similar to those of McCoy and McClung (1935). Rabbits were injected intravenously at two- to three-day intervals, the dose being increased from approximately .5 to 1.5 c.c. during the period of injection. Serum was stored at 10°C.(50°F.) without addition of preservative. A preliminary test showed a high titer (10,240 or more) for all of the sera.

Antigens for injection were obtained by growing the bacteria in tubes of liver broth containing liver scraps in the bottom, McClung and McCoy (1934). This medium gave the most stable suspensions of the rough strains which showed spontaneous agglutination in several other media tested. Twenty-four hour cultures were centrifuged, decanted, and resuspended in normal saline. Antigens for testing were grown and treated as above but were suspended in .4 per cent saline. The rough strains were shaken with beads to remove clumps. "O" (somatic) antigen was prepared by heating "W" (whole) antigen for two hours in flowing steam. Freshly prepared antigens were used for most of the tests but in some cases they were used after a week to ten-days' storage in the ice box, McCoy and McClung (1935). Agglutination and absorption technic and recording of results were those used by McCoy and McClung (1935).

Morphological and physiological studies of the pineapple clostridia had indicated that they were probably of the *Cl. pasteurianum* type. Agglutination tests with 17 cultures (7 of S, 10 of R type) were therefore carried out with sera prepared by McCoy against two strains of *Cl. pasteurianum* (Nos. 5, 7, McCoy, *et al.* 1930) and with

TABLE 5

Cross Agglutination of Smooth and Rough Types Using Whole (W) Antigens

Serum	W antigen	Serum dilutions									
		80	160	320	640	1280	2560	5120	10240	20480	40960
31(S)	9(R)	—	—	—	—	—	—	—	—	—	—
	10(R)	—	±	±	+	+	+	+	+	+	+
	35(R)	±	±	±	+	+	+	±	±	—	—
	47(R)	±	±	+	+	+	+	+	+	+	+
	57(R)	±	±	±	±	±	±	—	—	—	—
	82(R)	—	—	—	—	—	—	—	—	—	—
	30(S)	++	+++	+++	+++	+++	+++	++±	+++	+++	+++
	31(S)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
35(R)	9(R)	++±	+++	+++	+++	+++	+++	+++	+++	++	+
	10(R)	+++	+++	+++	+++	+++	+++	+++	+++	++±	+
	35(R)	++	+++	+++	+++	+++	+++	+++	+++	++	++
	47(R)	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
	57(R)	+++	+++	+++	+++	+++	+++	+++	+++	+++	++±
	82(R)	++	++	++	++	++	++	++	++	+±	+±
	30(S)	+±	±	—	—	—	—	—	—	—	—
	31(S)	+±	±	—	—	—	—	—	—	—	—

one serum for *Cl. beijerinckii* Donker strain. Titers for these three sera against homologous antigens were 20,480, 10,240, 5,120, respectively. With the 17 pineapple cultures, reactions were either entirely negative or positive only in low dilution (1:80) indicating group relationship only.

Cross agglutination tests with the pineapple clostridia against two sera, 31 (S) and 35 (R), show (Table 5) that the two types are serologically distinct. Some of the rough strains in smooth serum, however, showed some agglutination at high dilutions indicating a common factor for the two types. This is further indicated (Table 6)

TABLE 6

Cross Agglutination of Smooth and Rough Types Using Somatic (O) Antigens

Serum	O antigen	Serum dilutions							
		80	160	320	640	1280	2560	5120	10240
31(S)	9(R)	±	—	—	—	—	—	—	—
	10(R)	+	±	±	—	—	—	—	—
	35(R)	±	+	—	—	—	—	—	—
	47(R)	+	+	±	—	—	—	—	—
	57(R)	+±	+±	+	—	—	—	—	—
	82(R)	±	±	±	—	—	—	—	—
	30(S)	+	—	—	—	—	—	—	—
	31(S)	±	—	—	—	—	—	—	—
35(R)	9(R)	++±	+++	++±	++±	++±	+±	+	±
	10(R)	+	+	±	—	—	—	—	—
	35(R)	—	—	—	—	—	—	—	—
	47(R)	++	++	+	±	—	—	—	—
	57(R)	++	++	+±	+	+	±	—	—
	82(R)	++±	++	+	±	±	—	—	—
	30(S)	—	—	—	—	—	—	—	—
	31(S)	—	—	—	—	—	—	—	—

by cross reactions obtained with somatic antigens apparent only when rough strains were tested in serum for the smooth strain and not in the reciprocal cross.

In a further attempt to clarify the relationship between the smooth and rough types, absorption tests were resorted to. Two W sera (31 S and 35 R) were absorbed and tested with W and O antigens as shown (Table 7a and b). As expected, testing with O antigen gave negative results throughout the series (Table 7a). With W antigen, however, results were not clear cut. It is evident that the two sera were not completely absorbed by either homologous or heterologous antigens, although the homologous antigens removed more of the agglutinins than did the heterologous antigens. The rough strains (35

TABLE 7

Agglutination Following Absorption

A. W Absorption, Test With W and O Antigens

Serum	Abs. with W	Test with W	Serum dilutions 80	160	320	640	1280	2560	5120	10240	20480	40960	Test with O	Serum dilutions 80	160	320	640
31 (S)	31 (S)	31 (S)	++	++	++	+	—	—	—	—	—	—	31 (S)				
		35 (R)	±	±	±	±	—	—	—	—	—	—	35 (R)				
		57 (R)	—	—	—	—	—	—	—	—	—	—	57 (R)				
	35 (R)	31 (S)	—	+±	+++	+++	+++	+++	+++	+++	+±	+	31 (S)				
		35 (R)	±	±	±	—	—	—	—	—	—	—	35 (R)	All tubes in this series negative.			
		57 (R)	—	—	—	—	—	—	—	—	—	—	57 (R)				
35 (R)	31 (S)	31 (S)	++	++	++	+	—	—	—	—	—	—	31 (S)				
		35 (R)	+±	+±	+++	+++	+++	+++	+++	+++	+	±	35 (R)				
		57 (R)	+++	+++	+++	+++	+++	+++	++±	++	+±	±	57 (R)				
	35 (R)	31 (S)	±	+	±	±	—	—	—	—	—	—	31 (S)				
		35 (R)	±	+	+	±	—	—	—	—	—	—	35 (R)				
		57 (R)	+±	++	++	±	—	—	—	—	—	—	57 (R)				

B. O Absorption, Test With W and O Antigens

31 (S)	31 (S)	31 (S)	+++	+++	+++	+++	+++	+++	+++	+++	++±	+	31 (S)	—	—	—	—
		35 (R)	—	—	—	—	—	—	—	—	—	—	35 (R)	—	—	—	—
		57 (R)	—	—	—	—	—	—	—	—	—	—	57 (R)	—	—	—	—
	35 (R)	31 (S)	+++	+++	+++	+++	+++	+++	+++	+++	++±	+	31 (S)	±	—	—	—
		35 (R)	—	—	—	—	—	—	—	—	—	—	35 (R)	—	—	—	—
		57 (R)	—	—	—	—	—	—	—	—	—	—	57 (R)	—	—	—	—
35 (R)	31 (S)	31 (S)	+++	++	++	++	—	—	—	—	—	—	31 (S)	—	—	—	—
		35 (R)	+++	+++	+++	+++	+++	++	++	+±	—	—	35 (R)	—	—	—	—
		57 (R)	+++	+++	+++	+++	+++	+++	+++	+++	++	+	57 (R)	—	±	±	±
	35 (R)	31 (S)	++	++±	++	++	—	—	—	—	—	—	31 (S)	—	—	—	—
		35 (R)	+++	+++	+++	+++	+++	+++	+++	+±	+	—	35 (R)	—	—	±	±
		57 (R)	++	+++	+++	+++	+++	++	++	++	+	±	57 (R)	—	+	+	+

and 57) were nearly or completely negative in the serum for the smooth type (No. 31); however, the smooth strain (31) showed a fair titer in the serum for the rough strain (35), again indicating some factor common to the two types. This same relationship is shown even more strikingly (Table 7b) following absorption with O antigen and testing with W antigen. With serum for the smooth strain (31), agglutination was obtained only with the homologous antigen, but with serum for the rough strain (35), agglutination was obtained with both homologous and heterologous antigens.

While there are a number of inconsistencies in these results, the data in general lead to the conclusion that the two types of bacteria (S and R) dealt with are serologically dissimilar but have some factor or factors in common, indicating some degree of relationship. Physiologically the two types are identical, but since it has not been proved that they have been derived one from the other, the question remains a controversial one. However, the results of Orr, Josephson, Baker, and Reed (1933), Colef (1935), and Smith (1937) obtained with S and R strains of clostridia show that their serological reactions are not always clear cut. Orr, *et al.* found that a rough variant of *Cl. welchii* would not agglutinate in a serum prepared against the smooth strain of this same organism. Colef, on the other hand, obtained cross agglutination with both S and R strains of *Cl. oedematiens.* Smith, working with *Cl. histolyticum*, found that smooth strains "did not agglutinate with an antiserum prepared against a rough variant," although "rough variants, and certain strains of *Cl. sporogenes*, agglutinated with antisera to the normal smooth *Cl. histolyticum* or the rough variants."

IDENTIFICATION

In general, the bacteria here described are similar to *Cl. pasteurianum* as described by Winogradsky (1894), Omeliansky (1916), McCoy, *et al.* (1930), and Bergey (1939). Winogradsky reported this organism as not fermenting glycerol and mannitol, while our cultures do ferment these substances. In this respect our results agree rather with those of Omeliansky and with McCoy, *et al.*, whose culture No. 5 was obtained from Winogradsky. Winogradsky and Omeliansky both report that *Cl. pasteurianum* ferments dextrin, while our cultures and No. 5 of McCoy gave negative results. McCoy obtained positive results with culture No. 5 on arabinose, while our cultures and those described by Winogradsky and by Omeliansky gave negative results. McCoy also reports No. 5 positive on soluble starch, while our cultures gave negative results.

Sjolander and McCoy (1937) studied three cultures of so-called "*Cl. pasteurianum,*" and found that all of them produced stormy

fermentation of milk, digested corn mash, and produced appreciable quantities of solvents. In these respects their cultures were entirely different from our strains.

Cl. pasteurianum is separated from *Cl. beijerinckii*, Bergey (1939), because it fails to ferment lactose and mannitol. Our cultures differ from *Cl. beijerinckii* by utilizing glycerol but not lactose.

Van Beynum and Pette (1935) studied some 200 non-gelatin-liquefying, butyric anaerobes and divided them into two groups: *Cl. tyrobutyricum* and *Cl. saccharobutyricum*. The first group fermented glucose and lactate but was negative on sucrose, lactose, starch, xylose, mannitol, and glycerol. The second group fermented glucose, sucrose, lactose, starch, mannitol, and xylose and gave negative results on glycerol and lactate. Our cultures differ from their first group in regard to lactate, sucrose, mannitol, and glycerol and from the second group in regard to lactose, starch, and glycerol.

In addition to the two groups described by Van Beynum and Pette, Burri (1937) found a third group which combines the fermentation reactions of the other two. He states, however, that the fermentation reactions of the three groups are not constant enough to be used for purposes of classification.

In general, our cultures resemble those described by Townsend (1938, 1939) but his cultures differ from ours in showing lower optimum temperature for growth, greater heat resistance, and greater tolerance to acidity. Fermentation of carbohydrates also varied somewhat between his cultures and ours.

While the pineapple clostridia are not identical with previously described strains of *Cl. pasteurianum* (see particularly the serological data), they resemble this organism closely as described by Winogradsky (1894) and Omeliansky (1916). For the present, they will be considered as strains of this species. The grouping of *Cl. pasteurianum* Winogradsky with numerous other butyric-acid anaerobes into a single species, as done by Bredemann (1909) for *Bacillus amylobacter*, does not seem justifiable to the writer, but creation of new species because of minor differences seems equally uncalled for.

SUMMARY

Morphological and physiological characteristics are presented for saccharolytic butyric anaerobes isolated from bursting swells of canned pineapple. The two types studied are regarded as smooth and rough strains of one species, *Clostridium pasteurianum* Winogradsky.

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INFLUENCE OF FEEDS ON LECITHIN CONTENT OF MILK AND POSSIBLE RELATIONSHIP OF LECITHIN CONTENT TO SUSCEPTIBILITY OF MILK TO COPPER-INDUCED OXIDIZED FLAVOR[1, 2]

I. A. GOULD, W. K. FOX,[3] AND G. M. TROUT
Department of Dairying, Michigan State College, East Lansing, Michigan

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Although lecithin is considered one of the minor constituents of milk and milk products, it exerts an appreciable influence upon dairy procedures and processes in general; for example, this constituent is at least partially responsible for the stabilization of the fat/water emulsion in milk, for the fishy flavor of butter, and for certain errors which occur in the fat tests of dairy products, especially buttermilk.

Renewed interest in lecithin has arisen within the past few years, owing largely to the work of Thurston, Brown, and Dustman (1935) who found that the oxidation of this constituent is undoubtedly responsible for the oxidized flavor in milk. In general, verification of these results was secured by Chilson (1935), Dahle and Palmer (1937), and recently by Swanson and Sommer (1938).

Even though oxidation of lecithin results in an oxidized flavor in milk, this does not necessarily imply that variations in the lecithin content of milk may cause variations in the susceptibility of milk to oxidized-flavor development. If this were true, then milk with a large percentage of lecithin would be more easily oxidized than milk with a comparatively low percentage. Information is scarce relative to this possible relationship, although recent results by Beck, Whitnah, and Martin (1939) showed no correlation between the lecithin content of milk and the normal susceptibility of milk to oxidized-flavor development.

Another possibility in this connection involves the influence of feed and seasons on the prevalence of oxidized flavor. Many workers have observed the type of feed to influence markedly the oxidizability of the milk—Anderson, Hardenburg, and Wilson (1936); Brown, Thurston, and Dustman (1937); Brown, Van Landingham, and Weakley (1939); Chilson (1935); Guthrie and Brueckner (1934);

[1] This paper constitutes a portion of the thesis submitted by W. K. Fox to the faculty of Michigan State College in partial fulfillment of the requirements for the degree of Master of Science.

[2] Journal Article No. 383 (N.S.), Michigan Agricultural Experiment Station.

[3] Now of the Lansing, Michigan, Health Department.

Kende (1932); and Prewitt and Parfitt (1935)—and some have ascribed the seasonal variation in the prevalence of oxidized flavor, at least partly, to this factor. The role of lecithin in this connection has not been ascertained. In fact, practically no attention has been given to the possible influence of feeds on the lecithin content of milk.

Literature records few data relative to causes for variations in the lecithin content of milk. The most complete recent study of lecithin in milk is that of Horrall (1935). This author found the lecithin content of milk from a normal cow to be fairly constant after the first four days of the lactation period. Mastitis-infected cows were found to give milk with higher lecithin content than were normal cows.

The present study was conducted, therefore, with a view to determining the possible influence of feeds upon the lecithin content of milk, and also to ascertain if the susceptibility of the milk to oxidized flavor could be correlated with lecithin variations.

EXPERIMENTAL PROCEDURE

The milk used in the greater part of this experiment was secured from individual members of a group of eight cows containing two Brown Swiss, one Guernsey, two Holsteins, and three Jerseys.

This selection was made to secure a group of cows some of which were negative and others positive in regard to mastitis. Variations in the composition and type of feeds were made to determine if these variations influenced the lecithin content or susceptibility of the milk to oxidized-flavor development.

The first portion of the feed study consisted of a double feed-reversal experiment in which the level of the fat in the ration was the variant. One-half of the cows were placed on a high-fat ration and the remainder on a low-fat ration. Reversals were made after 24-day periods. All of the cows received a low-fat, basal ration of good-quality alfalfa hay, beet pulp, solvent-extracted soybean flakes, bone meal, and molasses. In addition, the high-fat group received soybean oil fed at a rate equal to the fat produced per cow per day.

After the final reversal period in the fat experiment, the low-fat group was placed on a ration of ground corn and alfalfa to determine if this change in ration would influence the factors being studied.

Finally, samples of milk were obtained from a group of four cows, one from each of the Jersey, Guernsey, Holstein, and Brown Swiss breeds. These samples were secured when the cows were on the balanced ration used for the entire college herd and later when this

ration was supplemented with pasture. These samples also were studied for lecithin variations and for oxidized flavor.

In all of the trials, unless otherwise indicated, samples of milk were collected at the noon milking every fourth day during the interim of the trials. These samples were obtained by hand milking into porcelain pails. The milk was immediately well-mixed, placed in bottles, and cooled.

Each sample of milk was divided into two portions. Copper was added to one portion as copper sulfate, at a rate sufficient to give a concentration of .6 p.p.m. of added copper in the milk. This concentration was deemed sufficient to cause susceptible milk to become distinctly oxidized. The portion of milk containing the copper was pasteurized in the bottle at 61.5°C.(142.6°F.) ± .5°C. for 30 minutes and was immediately cooled and stored at 4°C.(39.2°F.) for 48 hours. Following its storage the milk was examined for oxidized flavor by at least two experienced milk judges.

The other portion of the sample, the raw portion, was analyzed for lecithin. The method used was essentially that given by Horrall (1935) but was modified slightly in that the standard phosphate solution used for the colorimetric determination was prepared according to Bodansky (1932). Although the method utilized also includes other phospholipids in addition to lecithin, the results are expressed as lecithin.

The results were usually calculated and expressed both as percentage of lecithin in the milk and as percentage of lecithin in the fat. These methods of expression were utilized by Horrall (1935) and by Holm, Wright, and Deysher (1936).

DISCUSSION OF RESULTS

High-Fat and Low-Fat Rations: One hundred and sixty samples of milk were analyzed for lecithin and examined for oxidized flavor in the experiment dealing with high-fat and low-fat rations. These samples were collected at four-day intervals during the reversal periods and also at two-day intervals at the time of making the reversals.

Analysis of the data showed no relationship between changes in fat content of the feed and variations in lecithin content or in susceptibility of the milk to oxidation. The average lecithin content of the 80 samples secured when the cows were on the high-fat ration was .0474 ± .0009 per cent, as compared with .0461 ± .0011 per cent for the same number of samples taken when the cows were fed the low-fat ration. The average difference between the high-fat and low-fat samples is insignificant.

Comparatively wide fluctuations occurred in the lecithin content of milk from individual cows, these fluctuations apparently being uninfluenced by the ration. In no case was any indication given that the ration was affecting the lecithin percentage of the milk.

The susceptibility of the milk to oxidized flavor was also, in general, unaffected by the change in rations or changes in lecithin content of the milk. The differences which did occur appeared to be largely due to individual peculiarities of milk from different cows.

Distribution of the milk on the basis of lecithin percentage, and the relationship between percentage of lecithin in the milk and oxidized flavor are illustrated (Fig. 1). The curve illustrating the distribution of samples irrespective of the ration is based on analysis

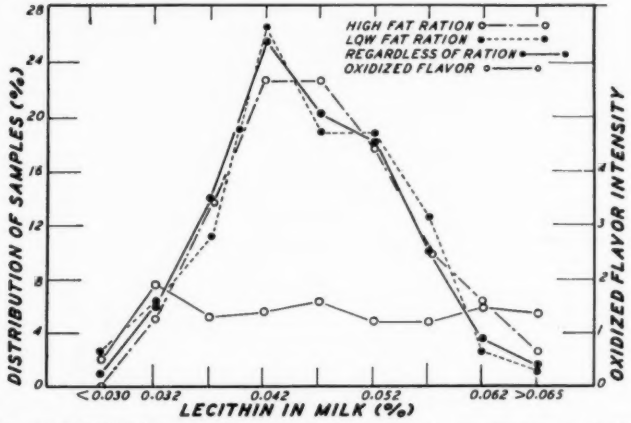

FIG. 1. Distribution of milk samples in respect to percentage of lecithin in the milk, and relationship of percentage of lecithin to oxidized flavor.

of all of the 198 samples of milk included in this study. The average percentage of lecithin for the 198 samples was .0460 ± .0006.

This graph shows that the distribution of samples on the basis of their lecithin content is not appreciably different for the different rations under consideration. In all cases, approximately 75 per cent of the samples contained from .037 to .052 per cent of lecithin. The figure also shows the total lack of correlation between percentage of lecithin in the milk and susceptibility of the milk to oxidized flavor. The milk was scored for flavor on the basis of 0 to 4, depending upon its intensity, i.e., 0 = no flavor, 4 = strong flavor. The graph shows the average oxidized-flavor score to be rather constant, with no apparent correlation to percentage of lecithin in the milk. Although this oxidized-flavor curve directly indicates the intensity of the fla-

vor, it also illustrates the prevalence of the defect. With such a large number of samples involved, any appreciable increase in frequency of occurrence of the flavor would also result in an increase in average score of the intensity.

In addition to expressing the results on the basis of percentage of lecithin in the milk, they were also expressed as percentage of lecithin in the fat (Fig. 2). On this basis, the average of the 80 samples secured from the cows on high-fat ration was .9892 ± .0311 per cent, as compared with an average of 1.0184 ± .037 per cent for milk secured from the cows on low-fat ration, a difference of only .0292 per cent. The average percentage of lecithin in the fat for the 198 samples included in this study was .9930 ± .0209.

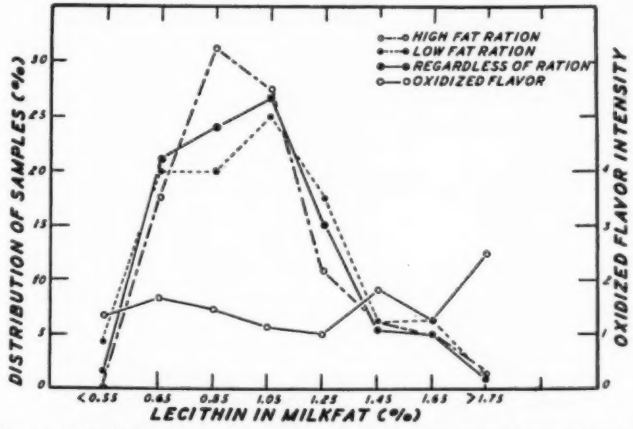

FIG. 2. Distribution of milk samples in respect to percentage of lecithin in the milk fat, and relationship of lecithin in the fat to oxidized flavor.

In general, a similarity is noticeable in distribution of the samples on the basis of percentage of lecithin in the fat, irrespective of the ration. There was a decidedly larger percentage, however, of the high-fat ration samples falling within the range of .85 to 1.05 per cent than there was of the samples secured while the cows were on other rations. A larger number of samples from the low-fat than from the high-fat ration gave values of approximately 1.25. These differences are not unexpected inasmuch as the high-fat ration may increase the fat content of the milk without altering the lecithin content. Thus the lecithin content based on percentage in the fat, may be lower in the high-fat ration samples.

No relationship is shown between intensity of the oxidized flavor in milk and percentage of lecithin in the fat (Fig. 2). The apparent

increase in intensity of the flavor shown at the lecithin values greater than 1.75 resulted, owing to the fact that only a few samples were involved and one showed pronounced oxidized flavor. A much larger group of samples in this class would have counterbalanced the influence of this one sample.

Miscellaneous Rations: Trials were also conducted in which cows were placed on a ground-corn and alfalfa ration, a normal well-balanced dry ration, and a normal well-balanced ration supplemented with pasture (Table 1). Since the cows which were on the low-fat rations were immediately transferred to the corn ration at the close of the fat studies, the low-fat results are included for comparison. The data presented are too limited to permit definite conclusions to be drawn; but, in general, the results show that the changes in ration did not noticeably affect the lecithin content of the milk. Differences

TABLE 1

Influence of Different Rations Upon Lecithin Content of Milk

Ration	Number of samples	Average lecithin content	
		In milk	In fat
		pct.	*pct.*
Low fat	80	.0461 ± .001	1.0184 ± .0372
Corn and alfalfa	15	.0411 ± .003	0.8802 ± .0641
Dry feed	12	.0430 ± .001	0.9831 ± .0647
Pasture	12	.0445 ± .001	0.9999 ± .0661

in individual samples from the same animal were far greater than any uniform change which may be attributed to the rations. In none of the comparative values (Table 1) are the differences significant.

The oxidized flavor was also not noticeably altered by changes in feeds, with the exception of the pasture trials. In these trials none of the four cows involved were producing susceptible milk at the close of the trials, whereas two had been producing such milk at the time they were transferred from dry to pasture feeding. As may be observed, the lecithin content of the milk was not appreciably different irrespective of whether the cows were on dry or pasture rations, again showing that the lecithin content in itself is not a factor in oxidized-flavor development.

Correlation of Data: The data secured on the 198 samples of milk indicate certain definite relationships between the lecithin content and the percentage of the fat (Fig. 3). These two lines of regression show the correlation between (a) the percentage of fat and percentage of lecithin in the fat, and (b) the percentage of fat and percentage of lecithin in the milk.

The regression line for the lecithin in milk and percentage of fat correlation shows that as the fat test of the milk goes up there is a tendency for the percentage of lecithin also to increase. The coefficient of correlation in this case was .3693 with a standard error of the coefficient of .0614. These values indicate a slight but significant correlation between the two variants.

The correlation between the fat test and the percentage of lecithin in the fat is highly significant. The coefficient of correlation is —.7406, and the standard error of the coefficient is .0321. This line of regression shows that as the lecithin on the fat basis increases, the fat test decreases. In other words, it would appear that the cow produces

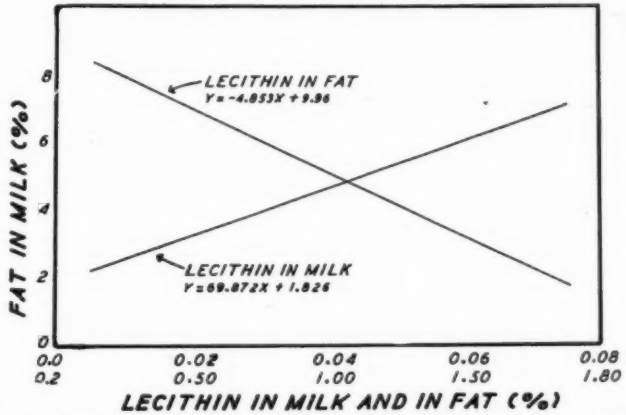

FIG. 3. Correlation between percentage of fat and percentage of lecithin in the milk or in the fat. (The lower values on the abscissa represent the lecithin in the fat, the upper values the lecithin in the milk.)

rather a constant amount of lecithin, irrespective of the amount of fat produced.

SUMMARY AND CONCLUSIONS

The results secured from analyzing 198 samples of milk for lecithin indicate that feeds do not influence the lecithin content of milk. High-fat and low-fat rations, corn and alfalfa, and a balanced ration with or without pasture supplement did not appreciably affect the lecithin content of the milk.

Eighty samples of milk secured when the cows were on a high-fat ration gave an average of .0474 ± .0009 per cent of lecithin in the milk and .9892 ± .0311 per cent of lecithin on the fat basis. An equal number of samples secured when a low-fat ration was fed averaged .0461 ± .0011 per cent of lecithin in the milk and 1.0184 ± .037 per cent of

lecithin in the fat. The average lecithin content of 198 samples of milk secured both from mastitis-infected and mastitis-free cows, and secured with no respect for the ration, was .0460 ± .0006 per cent for the milk and .9930 ± .0209 per cent when calculated on the basis of fat in the milk.

The lecithin values secured in this study are somewhat higher than those of Horrall (1935), Holm, Wright, and Deysher (1936), and Mohr, Brockmann, and Muller (1932) who reported percentages of .0276, .0337, and .0370, respectively, for the lecithin content of milk. Horrall found milk to average .852 per cent lecithin in the fat, and Holm, *et al.* reported a similar value of .0869. Other workers, however, have reported considerably higher values for the lecithin content of milk, with Chapman (1928) giving values of .345 to .709, and Mohr and Moos (1932) giving a range from .0038 to .2889 per cent.

Distribution curves developed from the data secured in this study show that approximately 75 per cent of the samples fall within the range of .037 to .052 on the basis of lecithin in the milk, and approximately 85 per cent within the range of .65 to 1.25 on the basis of lecithin in the fat.

A high negative correlation was found to exist between percentage of fat in the milk and percentage of lecithin in the fat; i.e., as the percentage of fat increased the percentage of lecithin in the fat decreased. This would indicate a fairly constant amount of lecithin in the milk irrespective of changes in the fat test. The coefficient of correlation was —.7406.

The correlation between percentage of lecithin in the milk and percentage of fat is much less significant than when the lecithin in the fat is considered. The coefficient of correlation of this relationship was .3693, a value which indicates only a slight tendency for the lecithin content of milk to increase with increases in the percentage of fat.

No relationship was observed between the susceptibility of milk to copper-induced oxidized flavor and the lecithin content. The individuality of the cows was apparently the most important factor in this connection, and rather wide variations in the percentage of lecithin in the milk could not be correlated with variations in susceptibility of the milk to oxidation.

The authors wish to express appreciation to Dr. C. F. Huffman for his assistance and coöperation relative to the feeding trials.

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A COMPARATIVE STUDY OF STANDARD LACTOSE BROTH AND MODIFIED EIJKMAN MEDIUM FOR ISOLATION OF *ESCHERICHIA COLI* FROM NUT MEATS

MORRIS OSTROLENK AND ALBERT C. HUNTER
U. S. Food and Drug Administration, U. S. Department of Agriculture, Washington, D. C.

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In 1933, Perry and Hajna studied the comparative values of a modified Eijkman medium and standard lactose broth for the isolation of *Escherichia coli* from fresh human feces. The results obtained from the examination of 23 specimens showed a greater recovery of *Escherichia coli* by the Eijkman method than with standard lactose broth. Subsequent studies by these investigators (1935) further indicated the superiority of the Eijkman medium over standard lactose broth in the examination of oysters, crabmeat, and other foods. They therefore suggested that in the examination of those products where *Escherichia coli* is the sole basis for estimating probable fecal pollution, the Eijkman medium be substituted for standard lactose broth.

The limitations of the Eijkman test for the detection of *Escherichia coli* from human feces was shown by Skinner and Brown (1934). Two-thirds of the samples examined by them contained thermolabile strains of *Escherichia coli* which were recovered with standard lactose broth but which failed to survive the incubation temperature of the Eijkman method.

Minkevich, Alexandrov, and Soboleva (1936), confirmed the findings of Skinner and Brown regarding the injurious influence on *Escherichia coli* of 46°C.(114.8°F.) incubation. The data obtained by these investigators indicated that 43.5°C.(110.3°F.) accelerated multiplication and gas production by *Escherichia coli* and that, at this temperature, incubation could be limited to 24 hours.

Bartram and Black (1937a), in studies on the reaction in Eijkman medium of coliform bacteria isolated from milk, found that although none of their Aerobacter and Citrobacter strains produced gas in this medium in 48 hours, nine of the 13 strains of Escherichia likewise failed to give positive presumptive reactions. Attempts by these authors (1937b), to use the Eijkman method for the isolation of coliform bacteria from milk were not successful.

More recent studies by Payne (1938) on the comparative values of these two media in the examination of oysters, clams, and shellfish-growing waters showed that standard lactose broth gave con-

sistently greater recovery of *Escherichia coli* than did the Eijkman medium. She found that transplants from positive presumptive Eijkman broth tubes to eosin methylene blue agar resulted in the formation of atypical colonies. Transfers from such colonies, when repeatedly inoculated into lactose broth incubated at 37°C.(98.6°F.) and then streaked on eosin methylene blue agar, developed typical Escherichia or Aerobacter colonies.

In the examination of foodstuffs for the presence of *Escherichia coli* it is necessary that there be a correlation of survey data with laboratory results in order to evaluate properly the significance of the presence of various members of the coliform group. The detection of fecal pollution by bacteriological means is often made difficult by the possible attenuation or dying-off of the contaminants over long periods of storage. In food products where these factors are encountered any method which would enhance the recovery of *Escherichia* coli would be desirable.

In a recent study by Ostrolenk and Hunter (1939) examination of 548 samples of nut meats, using the standard lactose broth method, resulted in the recovery of *Escherichia coli* in a substantial number of samples. To compare the relative merits of the Eijkman medium and standard lactose broth a series of 114 samples of nut meats were examined by both methods.

EXPERIMENTAL PROCEDURE

The samples were collected aseptically in sterile pint glass or tin containers during the course of a sanitary survey of pecan shelling. The samples represented stored material and specimens collected at practically every stage in the shelling operations. A minimum of 21 days and a maximum of 42 days elapsed between the date of collection and the date of examination.

Fifty grams of pecan meats were washed with an equal amount of sterile distilled water. Inoculations were made simultaneously into standard lactose broth and Eijkman media in amounts varying from 10 c.c. to 1/1000 c.c., representing washings of from 10 to 1/1000 gram of pecan meats. Incubation of the lactose broth was at 37°C. (98.6°F.). The Eijkman broth tubes were incubated in a water jacketed incubator at 46°C.(114.8°F.). The maximum temperature fluctuation was ±.5°C. during the course of the experiment. Transfers from tubes in which presumptive tests were obtained were streaked on Levine's eosin methylene blue agar (Difco) at the end of 24 and/or 48 hours. Well-isolated colonies were fished directly into tryptophane broth, methyl red-Voges Proskauer media, and Koser's citrate broth for biochemical determinations. Cultures which

were methyl red positive, Voges Proskauer negative, and citrate negative were recorded as *Escherichia coli* regardless of their ability or inability to produce indol.

The following modified Eijkman formulae suggested by Perry and Hajna (1933) were used:

Ingredient	FORMULA II	III
Dextrose	3.6 grams	6 grams
Peptone (Difco)	18.0 grams	30 grams
K_2HPO_4	4.8 grams	8 grams
KH_2PO_4	1.8 grams	3 grams
NaCl	6.0 grams	10 grams
Distilled water	1,000 c.c.	1,000 c.c.
	(final pH 6.8 to 6.9)	

The initial 10 c.c. of inoculum representing washings of 10 grams of pecans were introduced into tubes containing broth prepared according to formula No. III. All subsequent inoculations of washings from one to one-thousandth gram portions were made into tubes containing broth made up in accord with formula No. II.

DISCUSSION OF RESULTS

When using standard lactose broth at least one sample from each of 14 pecan-shelling plants yielded evidence of the presence of *Escherichia coli*. By the Eijkman method it was not possible to demonstrate evidence of fecal pollution in samples from four of the 14 plants. Moreover, in two of the 10 plants in which fecal pollution was detected by the Eijkman method, *Escherichia coli* was disclosed in an equal number of samples by both Eijkman medium and lactose broth, while in eight plants *Escherichia coli* was recovered in a higher number of samples by the use of lactose broth than by the use of Eijkman medium. Based on the total number of samples examined, the use of standard lactose broth yielded 15.7 per cent greater recovery of *Escherichia coli* than did the use of Eijkman medium (Table 1).

The comparative values of these two media are demonstrated, based on the results obtained from the individual inoculated tubes (Table 2). Presumptive tests developed in more than three times as many lactose broth tubes as Eijkman broth tubes. The percentage of individual tubes containing *Escherichia coli* are calculated on the basis of the total number of tubes as well as on the basis of the number of tubes which showed the presence of gas. Based on the first consideration, total tubes inoculated, *Escherichia coli* was isolated from six per cent more lactose broth tubes than from a corresponding number of Eijkman broth tubes. When the percentage is computed on the basis of tubes showing gas, 21 per cent of the positive

TABLE 1

Number of Plants and Samples From Which Escherichia coli Was Recovered

Plant No.	Number of samples	*Escherichia coli* recovered in			
		Plants		Samples	
		Lactose	Eijkman	Lactose	Eijkman
1	13	1	1	2	1
2	10	1	1	3	2
3	7	1	1	5	4
4	5	1	0	1	0
5	9	1	0	2	0
6	10	1	1	4	1
7	6	1	1	5	4
8	8	1	1	4	4
9	7	1	1	6	3
10	6	1	1	3	1
11	6	1	0	1	0
12	11	1	1	3	3
13	9	1	0	1	0
14	7	1	1	4	3
—	—	—	—	—	—
Total:					
Plants 14	....	14[1]	10[2]	....	....
Samples	114	....	....	44[3]	26[4]

[1] 100 per cent recovery. [2] 71.4 per cent recovery. [3] 38.5 per cent recovery. [4] 22.8 per cent recovery.

TABLE 2

Comparative Results Based on Total Number of Tubes Inoculated and Those in Which Presumptive Reactions Were Obtained

	Lactose		Eijkman	
Number of tubes inoculated.....	570		570	
Number of tubes presumptive.....	374		118	
Number of tubes *Escherichia coli*....	78 (14%)[3]	(21%)[4]	48 (8%)[3]	(41%)[4]
Number of tubes group[1].....	244	(65%)[4]	42	(36%)[4]
Number of tubes negative[2].....	52	(14%)[4]	28	(23%)[4]

[1] By "group" is meant members of colon-aerogenes group, exclusive of *Escherichia coli*. [2] By "negative" is meant presumptive reactions owing to non-coliform bacteria. [3] Per cent of inoculated tubes. [4] Per cent of tubes showing presumptive reaction.

presumptive lactose broth tubes were confirmed as *Escherichia coli*; whereas, in 41 per cent of the Eijkman broth tubes in which gas developed the coliform organisms present were confirmed as *Escherichia coli.* Twenty-nine per cent more standard lactose broth tubes contained coliform bacteria not confirmed as *Escherichia coli* than did Eijkman presumptive tubes. The standard lactose broth tubes developed nine per cent more false presumptive reactions than did the Eijkman broth tubes.

SUMMARY

Use of the Eijkman method for the examination of pecans failed to enhance the recovery of *Escherichia coli.* Although the Eijkman medium surpassed standard lactose broth in limiting the development of Aerobacter and Citrobacter, it reduced the development of Escherichia. Three times as many standard lactose broth tubes developed presumptive tests as did a corresponding number of inoculated Eijkman broth tubes. In determining evidence of fecal pollution on pecan meats standard lactose broth yielded *Escherichia coli* in 28.6 per cent more plants, 15.7 per cent more samples, and six per cent more inoculated tubes than did the Eijkman medium.

In addition to the less-favorable bacteriological results obtained by the Eijkman method its limiting temperature requirements greatly influence its practicability in routine laboratory practice.

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BACTERICIDAL PROPERTIES OF ALLYL ISOTHIOCYANATE AND RELATED OILS

MILTON J. FOTER
Department of Bacteriology, University of Connecticut, Storrs, Connecticut

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Walton, Herbold, and Lindegren (1936) and Lovell (1937) showed that the vapors from crushed garlic and onions contained bactericidal substances. Foter and Golick (1938) reported the same property for the vapors from crushed horse-radish. Vollrath, Walton, and Lindegren (1937) and Ingersoll, Vollrath, Scott, and Lindegren (1938) attributed the bactericidal effects of the vapors from crushed garlic and onions to acrolein and crotonaldehyde.

In an attempt to attribute the inhibitory properties of the vapors from crushed horse-radish to some definite compound or compounds, a study was made of the literature relative to its composition. Chemical analyses of crushed horse-radish reported by Gildemeister and Hoffmann (1916) and by Heiduschka and Zwergal (1931) show the main volatile constituent to be mustard oil, allyl isothiocyanate (CH_2=$CHCH_2NCS$), 20 to 25 per cent phenyl ethyl isothiocyanate (C_6H_5—CH_2CH_2NCS), and a trace of phenyl propyl isothiocyanate (C_6H_5—$[CH_2]_3NCS$). This report is concerned with a study of the bactericidal effects of allyl isothiocyanate and two other oils similar in structure, namely, methyl isothiocyanate (CH_3NCS) and ethyl isothiocyanate (CH_3CH_2NCS). An attempt is being made at the present time to synthesize the other isothiocyanates reported in crushed horse-radish.

EXPERIMENTAL PROCEDURE

The bactericidal properties of the oils were studied by three different methods, using 10 test organisms.

Method 1: Agar plates were poured using approximately 15 ml. of a suitable medium and the agar was allowed to solidify. The plates were inverted and sterile filter paper placed in the tops. Various amounts of the oils were placed on the filter paper, the plates were sealed, and the agar was exposed to the vapors from the oils at 37°C.(98.6°F.) for varying lengths of time (Table 1). After the exposure periods the covers of the Petri plates were removed and replaced by sterile tops and immediately streaked, using a standard four-mm. loopful of a 24- to 48-hour broth culture of the test organism as the inoculum. The plates were then resealed and incubated

TABLE 1

Growth Score of Organisms on Agar Plates After Exposure to Oil Vapors

Organism	Oil	Allyl isothiocyanate						Methyl isothiocyanate						Ethyl isothiocyanate					
		0	8[2]	16	32	60	120	0	8	16	32	60	120	0	8	16	32	60	120
	ml.																		
Serratia marcescens	.1	4	0[3]	0	0	0	0	4	0	0	0	0	0	4	0	0	0	0	0
	.01	4	0	0	0	0	0	4	0	0	0	0	0	4	4	3.5	3	3	2.5
	.001	4	4	3	3	3	2	4	4	4	3	2	1	4	4	4	4	4	4
	.0001	4	4	4	4	4	3	4	4	4	4	4	4	4	4	4	4	4	4
Bacillus subtilis	.1	4	0	0	0	0	0	4	0	0	0	0	0	4	0	0	0	0	0
	.01	4	0	0	0	0	0	4	0	0	0	0	0	4	4	3	3	3	2
	.001	4	4	4	3	2.5	2	4	4	3	2.5	2	1	4	4	4	3.5	3	3
	.0001	4	4	4	4	3	3	4	4	4	4	4	4	4	4	4	4	3.5	3
Escherichia coli	.1	4	0	0	0	0	0	4	0	0	0	0	0	4	0	0	0	0	0
	.01	4	2	2	1	1	0	4	0	0	0	0	0	4	4	4	4	4	4
	.001	4	4	4	3	3	2	4	4	4	4	4	4	4	4	4	4	4	4
	.0001	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Bacillus mycoides	.1	4	0	0	0	0	0	4	0	0	0	0	0	4	0	0	0	0	0
	.01	4	2	0	0	0	0	4	0	0	0	0	0	4	4	2	2	2	2
	.001	4	4	4	4	3.5	3	4	3	2.5	1	1	0	4	4	4	4	4	4
	.0001	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
MRX-1[1]	.1	4	0	0	0	0	0	4	0	0	0	0	0	4	0	0	0	0	0
	.01	4	0	0	0	0	0	4	0	0	0	0	0	4	2	2	2	2	2
	.001	4	4	3	3	2	1	4	4	4	4	3.5	3	4	3.5	3	2.5	2	2
	.0001	4	4	4	4	4	4	4	4	4	4	4	3	4	4	4	4	4	4
Eberthella typhosa	.1	4	0	0	0	0	0	4	0	0	0	0	0	4	0	0	0	0	0
	.01	4	0	0	0	0	0	4	0	0	0	0	0	4	4	4	4	4	4
	.001	4	4	4	4	4	4	4	4	3.5	2.5	3	1.5	4	4	4	4	4	4
	.0001	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Staphylococcus aureus	.1	4	0	0	0	0	0	4	0	0	0	0	0	4	0	0	0	0	0
	.01	4	4	4	4	4	4	4	0	0	0	0	0	4	4	4	4	4	4
	.001	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	.0001	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Mycobacterium tuberculosis var. *hominis*	.1	4	0	0	0	0	0	4	0	0	0	0	0	4	0	0	0	0	0
	.01	4	4	4	3	3	3	4	0	0	0	0	0	4	4	4	4	4	4
	.001	4	4	4	4	4	3	4	4	4	4	4	4	4	4	4	4	4	4
	.0001	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Mycobacterium tuberculosis var. *bovis*	.1	4	0	0	0	0	0	4	0	0	0	0	0	4	0	0	0	0	0
	.01	4	3	3	3	0	0	4	0	0	0	0	0	4	4	4	4	4	4
	.001	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	.0001	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Mycobacterium phlei	.1	4	0	0	0	0	0	4	0	0	0	0	0	4	0	0	0	0	0
	.01	4	3	2	2	1	1	4	0	0	0	0	0	4	4	3.5	3	3	1
	.001	4	4	4	3	3	3	4	4	4	4	4	4	4	4	4	4	4	4
	.0001	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

[1] Isolated from cases of rat pneumonia. Probably identical with *Bacillus bronchisepticus*. [2] The number at the top of each column indicates the time of exposure in minutes. [3] Each number represents an average of two to three experiments.

at 37°C. for at least a week. Controls were made in all experiments.

A comparative scale was used in estimating the amount of growth after the incubation period by which 4 indicated full growth as in the controls, 3 indicated from three-fourths to full growth, 2 indicated one-half to three-quarters of full growth, and 1 indicated any growth to one-half of full growth.

Method 2: Dilutions of the oils, from 1:100 to 1:1,000,000, were prepared in a suitable agar medium. Plates were poured and streaked, using again a standard four-mm. loopful of a 24- to 48-hour broth culture of the test organism. The plates were sealed and incubated at 37°C. for one week, after which they were scored for growth as described above.

Method 3: In order to determine the approximate times required for killing and the effect of the presence of organic matter on bactericidal efficiency of the oils, two series each of aqueous suspensions of the oils were prepared in dilutions from 1:000 to 1:1,000,000. One series contained five per cent by volume of sterile cow serum. The aqueous suspensions were brought to a temperature of 37°C., after which each tube containing 10 ml. was inoculated with .1 ml. of a 24- to 48-hour broth culture of the test organism. The cotton plugs were then replaced with sterile rubber stoppers to prevent loss of the oils by vaporization and the tubes were incubated at 37°C. At various time intervals (.5, 1, 2, 4, 6, 8, 12, 24, 48, and 72 hours) standard four-mm. loop transfers were made from the dilutions to suitable sterile broth in tubes. These in turn were reincubated for at least a week before the results were recorded. Inoculated sterile distilled water controls with and without cow serum were made.

Attempts were made to prepare emulsions of the oils rather than suspensions, using various emulsifying agents with little success. Most of the emulsifying agents were themselves bactericidal for the test organisms. In all the methods where the growth of the test organism in broth did not yield uniform suspensions of cells for inoculations, the broth culture was transferred to a sterile glass-stoppered bottle containing glass beads, shaken vigorously, and filtered through sterile cotton.

RESULTS

The bactericidal effects of vapors from the volatile oils tested (Table 1) show that complete or marked inhibition of growth resulted in most cases when agar plates were exposed to the vapors from .1 ml. or .01 ml. of the oil for as short a time as eight minutes. Vapors from allyl isothiocyanate, the main volatile constituent found in crushed horse-radish, inhibit the growth of microörganisms in a manner similar to the vapors from crushed horse-radish itself. The vapors from

methyl isothiocyanate appear to be the most effective, those from ethyl isothiocyanate the least effective.

The bactericidal efficiency of the oils was more evident when exact dilutions were prepared in agar (Table 2). Complete or marked inhibition of growth of the test organisms resulted in most cases from dilutions as high as 1:100,000 and 1:1,000,000. Ethyl isothiocyanate was again the least effective of the oils.

Three test organisms were employed in the study of the oils by Method 3, *Escherichia coli*, *Bacillus subtilis*, and *Mycobacterium tuberculosis* var. *hominis*. In practically all cases the results confirm those obtained by Method 2. *Escherichia coli* was the most resistant test organism requiring a 24- to 48-hour exposure to a 1:10,000 dilution of the oils to obtain complete killing. Growth of the human strain of *Mycobacterium tuberculosis* was completely inhibited by a 48- to 72-hour exposure to dilutions of 1:100,000 or 1:1,000,000. *Bacillus subtilis* was the least resistant requiring only an eight- to 12-hour exposure to a 1:1,000,000 dilution of allyl and methyl isothiocyanate to obtain complete inhibition of growth. In all cases exposure to the 1:100 dilution of the oils for the shortest time inter-

TABLE 2

Bactericidal Effects of Oils Diluted in Agar

Organism	Allyl isothiocyanate						Methyl isothiocyanate						Ethyl isothiocyanate					
	Control	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000	Control	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000	Control	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000
Serratia marcescens	4	0[1]	0	0	0	0	4	0	0	0	0	0	4	0	0	0	0	0
Bacillus subtilis	4	0	0	0	0	0	4	0	0	0	0	0	4	0	0	0	0	1
Escherichia coli	4	0	0	1	1	1	4	0	0	0	0	1	4	0	0	2	4	4
Bacillus mycoides	4	0	0	0	0	0	4	0	0	0	0	0	4	0	0	0	0	0
MRX-1	4	0	0	0	3	4	4	0	0	0	0	0	4	0	0	0	4	4
Eberthella typhosa	4	0	0	0	0	0	4	0	0	0	0	0	4	0	0	0	0	0
Staphylococcus aureus	4	0	0	1	2	3	4	0	0	1	1	2	4	0	2	3	4	4
Mycobacterium tuberculosis var. *hominis*	4	0	0	0	0	2	4	0	0	0	0	0	4	0	0	0	0	1
Mycobacterium tuberculosis var. *bovis*	4	0	0	0	1	2	4	0	0	0	0	0	4	0	0	0	0	1
Mycobacterium phlei	4	0	0	0	0	1	4	0	0	0	0	0	4	0	0	0	0	0

[1] Each number represents an average of three to four experiments.

val, one-half hour, resulted in complete destruction of the cells. The presence of the five-per cent sterile cow serum slightly inhibited the bactericidal efficiency of the oils.

DISCUSSION

The apparent discrepancy in efficiency of the oils on the test organisms when agar plates are exposed to their vapors and when various dilutions of the oils are prepared in agar or aqueous suspensions may best be explained by the fact that when agar plates are exposed to the vapors, the oil is at first concentrated at the surface. During incubation the oils diffuse through the agar and concentration at the surface becomes too low to inhibit growth.

The test organisms employed differ in their resistance to the oils. *Escherichia coli* and *Staphylococcus aureus* appear to be the most resistant; while *Serratia marcescens*, *Bacillus subtilis*, and *Bacillus mycoides* are the least, with the other test organisms between these.

CONCLUSIONS

1. Allyl isothiocyanate, the main volatile constituent found in crushed horse-radish, and the related compounds, methyl and ethyl isothiocyanate, exhibit bactericidal effects on a variety of organisms when tested by several methods. The organisms employed differ in their resistance to these compounds.

2. The bactericidal efficiency of the oils may be more accurately determined by dilution in agar or by the preparation of aqueous suspensions than by exposure of agar plates to the vapors from similar dilutions.

3. The presence of five per cent by volume of sterile cow serum slightly interferes with the bactericidal efficiency of the oils.

4. Allyl isothiocyanate is a pungent volatile oil which irritates the eyes and burns the skin. Its presence in crushed horse-radish, and identification by several workers, probably explains the characteristic effects obtained when handling this substance and also the inhibitory properties of its vapors on microörganisms.

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INFLUENCE OF SUNLIGHT ON FLAVOR AND ASCORBIC ACID CONTENT OF MILK EXPOSED IN THREE DIFFERENT TYPES OF PAPER CONTAINERS

J. L. HENDERSON, D. C. FOORD, AND C. L. ROADHOUSE
Division of Dairy Industry, University of California, Davis, California

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Single-service paper containers for milk are being used more widely each year. The effect of sunlight on the flavor of milk stored in such containers has not, however, been studied extensively; and few data are available in the literature on this subject.

REVIEW OF LITERATURE

Doan and Myers (1936) studied the influence of sunlight on milk exposed in clear glass, in opaque glass, and in one brand of paper container (Sealcone). The reduction time of sterilized methylene blue milk in such containers was used as an index of the amount of light penetrating the container. With skim milk they found the reduction times were identical, whereas with whole milk the opaque glass gave complete protection and the paper container partial protection. With clear glass the reduction time was greatly decreased. The effect on milk flavor was that the milk exposed in paper containers became slightly tallowy after 30 minutes in the sunlight and definitely tallowy after 50 minutes. The milk in the clear glass bottles had a slight burnt flavor after 20 minutes and a burnt, slightly tallowy flavor after 30 minutes. Tracy (1938), studying another type of bottle (Pure-pak), concluded, "While milk stored in paper containers will acquire the 'sunshine flavor,' the effect of the sun is not nearly so serious as it is in the case of the milk in glass bottles." He gave no data, however.

Guthrie, Hand, and Sharp (1939) investigated the stability of added ascorbic acid in milk exposed to sunlight in one type of paper container. "Only a slight decrease in ascorbic acid occurred," they state, "in the samples held in paper bottles. This indicated that the paper bottle protected appreciably the milk from the accelerative effect of sunlight on the destruction of ascorbic acid in the presence of oxygen. In addition it was observed that the paper bottle, to some extent, protected the milk against the development of the burnt, or oily, or sunlight flavor."

EXPERIMENTAL PROCEDURE

Source of Milk: The milk used for determining the effect of sunlight upon rate of ascorbic acid oxidation and upon milk flavor was

so obtained as to prevent uncontrolled exposure to light and contact with copper. The milk samples, secured from three breeds of dairy cattle—Holsteins, Jerseys, and Ayrshires—were pasteurized in a stainless steel vat, cooled to 4.4°C.(40°F.), and put into the containers for exposure to sunlight. Control samples in each type of container were stored in the dark at 40°F.

Exposure of Milk to Sunlight: Throughout the experiments, which were conducted between 11 a.m. and 1 p.m. the light was measured with a Weston Photometer and was found to be practically constant over successive days. The containers exposed to the sunlight were placed in a row upon a white parchment paper in order that the reflection might be constant for the different experiments.

The milks were kept in the sunlight for periods ranging, in the different experiments, from 30 minutes to two hours. After this exposure the containers were stored at 40°F. with the controls. The milks were scored for flavor at two and at 20 hours after the end of the exposure periods.

Scoring Milk: In order to remove psychological bias from the experiment, the milks were placed in half-pint glass bottles, keyed as unknowns, and placed in random order for scoring. The controls and the exposed milks were scored by three judges, using the score card of the American Dairy Science Association. Since the purpose of scoring was to identify and evaluate the effect of sunlight upon the flavor, a score of 23 was given to samples in which the "sunshine flavor" could not be detected. The scores on the other samples were cut by the judges to indicate their opinion as to the extent of the flavor defect.

Ascorbic Acid Determination: Ascorbic acid was determined by a modified Bessy and King procedure. A trichloracetic acid serum of the milk was titrated with 2.6 dichlorobenzene indophenol solution. Metaphosphoric acid was added to the trichloracetic acid solution, according to Lyman, Schultze, and King (1937). The dye was stabilized by replacing 10 per cent of the double distilled water with a pH 6.8 phosphate buffer. The strength of the dye was determined by the thiosulfate method of Menaker and Guerrant (1938).

The trichloracetic acid was added to 25 ml. of the samples of the milks immediately at the end of the exposure periods.

Types of Paper Containers Studied: As was shown by a review of the literature, different workers using a single type of paper container secured results at variance with one another.

In this study the effect of sunlight on flavor and ascorbic acid content of the same milks was compared for three types of paper milk-containers now in general use throughout the United States.

Container A is of white bleached paper, the thinnest of the three. Container B is a cream-colored paper, thicker than A but thinner than C. Container C appears white because the outer plies of the paper are bleached; but the inner plies are unbleached and light brown. It is the thickest of the three.

The paraffin of all three types was applied between 73.9 and 76.7°C.(165 and 170°F.).

RESULTS

Influence of Sunlight on Ascorbic Acid Destruction and Flavor of Milk: A number of workers—Guthrie, Hand, and Sharp (1939) and Kon and Watson (1937)—have shown how sunlight affects the destruction of ascorbic acid in milk held in glass containers. The

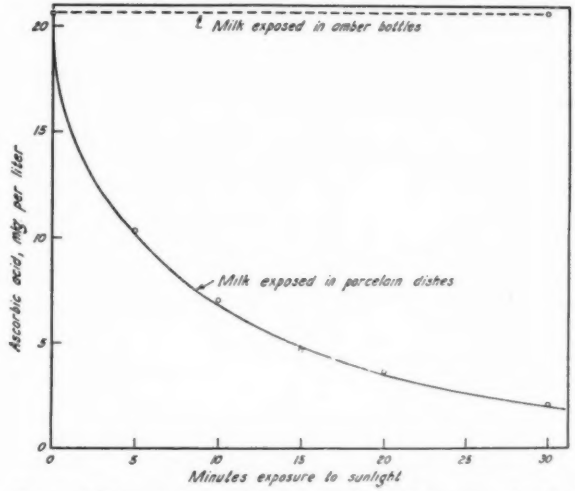

FIG. 1. Influence of sunlight on ascorbic acid content of milk.

effect is shown here (Fig. 1) of exposing milk in open-faced porcelain dishes for short periods of time to direct action of the sunlight. The milk was so produced and handled as to avoid uncontrolled exposure to light and contact with copper.

As is manifest (Table 1 and Fig. 1), there is a close relation between the effect of sunlight on the flavor of milk and the destruction of ascorbic acid. In the study of milk exposed in paper containers the destruction of ascorbic acid was used as an index of the amount of light penetrating the paper.

Influence of Sunlight on Oxidation of Ascorbic Acid in Milk Held in Glass and Paper Containers: How sunlight affects the destruction

of ascorbic acid in milk exposed for 30 and 120 minutes in clear glass and in three different types of paper milk-containers is shown (Fig. 2). Evidently all the paper containers permitted less light to penetrate into the milk than did the clear glass bottles, but they varied markedly in their ability to protect the milk from the action of sunlight on the ascorbic acid.

Influence of Sunlight on Flavor of Milk Exposed in Paper Containers: Ten trials were made with milk from different cows. The results were uniform in that the three paper containers were always placed in the same order with respect to their protective action against the extent of development of "sunshine flavor" in milk (Table 2).

The scoring order indicates the arrangement of the samples as

TABLE 1

Influence of Sunlight on Flavor of Milk

Sample	Scoring order	Judges' scores and comments			
		C. L. R.		J. L. H.	
		Score	Comment	Score	Comment
Control; no sunlight	1	23+	O.K.	23	O.K.
5 min. in sunlight	3	22.5	Not oxidized	23—	O.K.
10 min. in sunlight	7	21.0	Sunshine flavor	22.0	Slight sunshine flavor
15 min. in sunlight	2	21.0	Sunshine flavor	20.5	Sunshine flavor
20 min. in sunlight	6	19.5	Sunshine flavor	19.0	Sunshine flavor
30 min. in sunlight	4	19.0	Sunshine flavor	19.5	Sunshine flavor
30 min. in sunlight (in an amber bottle)	5	23.0	O.K.	23.0	O.K.

unknowns for scoring. When the data are tabulated by treatment rather than by scoring order, the results of the three judges are seen to be uniform. The controls all received a score of 23 and were not criticized. The milks exposed for one hour showed that the milk in Container A had a slight "sunshine flavor"; the milk in Container C was not affected; while the milk in Container B, although slightly different in flavor, was not characterized as "sunshine." The milks exposed for two hours gave similar results except that the flavor of the milk in Container A became more pronounced. In no cases were the milks exposed in paper containers so badly affected by the sunlight as those in clear glass bottles. These results place the containers in the order of increasing effect on milk flavor—C, B, A. The results shown for the effect of sunlight on the destruction of ascorbic acid places them in the same order (Fig. 2).

To indicate the relative amount of light passing through the different types of paper containers, a Weston Photometer was placed

TABLE 2

Influence of Sunlight on Flavor of Milk Exposed in Clear Glass Bottles and Paper Containers

Sample	Container	Scoring order	Judges' scores and comments					
			J. L. H.		C. L. R.		D. C. F.	
			Score	Comment	Score	Comment	Score	Comment
Controls not exposed to sunlight	Glass	12	23	O.K.	23	O.K.	23	O.K.
	Container A	6	23	O.K.	23	O.K.	23	O.K.
	Container B	9	23	O.K.	23	O.K.	23	O.K.
	Container C	2	23	O.K.	23	O.K.	23	O.K.
One hour in sunlight	Glass	7	19	Sunshine flavor	20.5	Sunshine flavor	20	Sunshine flavor
	Container A	11	22	Slight sunshine flavor	22.5	Slight sunshine flavor	22	Slight sunshine flavor
	Container B	8	23	Slightly different	23	Slightly different	23	O.K.
	Container C	1	23	O.K.	23	O.K.	23	O.K.
Two hours in sunlight	Glass	4	17	Sunshine flavor	18	Sunshine flavor	19	Sunshine flavor
	Container A	5	20	Sunshine flavor	21	Slight sunshine flavor	21	Sunshine flavor
	Container B	3	23	Slightly different	23	Slightly different	23	O.K.
	Container C	10	23	O.K.	23	O.K.	23	O.K.

behind an unprinted surface of the container, with a constant light source on the other side. The following readings were thus secured: Container A, 400; Container B, 180; Container C, 80. Although these values indicate that different amounts of light of certain wave lengths do penetrate the paper container, it does not necessarily follow that the effect of the sunlight on milk flavor, exposed in the different containers, is proportional to the relative readings shown on the photometer.

DISCUSSION OF RESULTS

According to the results, all the paper containers gave greater protection from the effects of sunlight on milk flavor and ascorbic

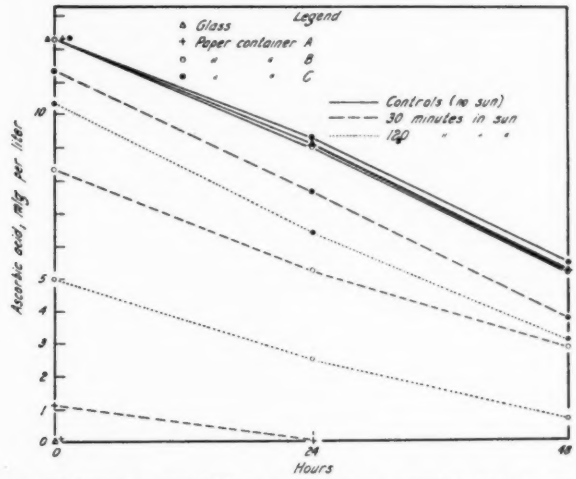

FIG. 2. Influence of sunlight on oxidation of ascorbic acid in milk held in clear glass and in paper milk-containers.

acid oxidation than did the clear glass bottles. The paper containers varied markedly among themselves in their protective action, which is apparently related to the color, structure, and thickness of the paper stock. Container C, with the colored inner plies and the thicker paper, protected the milk against "sunshine flavor" when exposed for two hours and also against most of the effects on the ascorbic acid oxidation.

The paper milk-containers would probably be more satisfactory from the standpoint of flavors if the paper stock contained unbleached or colored plies. Judging from this study, the rate of destruction of ascorbic acid in milks, exposed to the sunlight in different types of

paper containers, may be used to evaluate their relative abilities to exclude the objectionable sun rays.

SUMMARY AND CONCLUSIONS

Three types of paper milk-containers of different paper stocks were compared for protective effects against the flavor production and ascorbic acid destruction caused by exposure to sunlight.

All the paper containers were found to exhibit greater protection from the effects of the light rays than did clear glass milk bottles.

Paper containers studied varied greatly among themselves in their ability to protect the milk from the development of "sunshine flavor."

The container made of thick paper with unbleached or colored inner plies gave complete protection against the flavor defect and nearly complete protection against ascorbic acid destruction.

The effect of sunlight on ascorbic acid destruction was found to be useful as an index of the effect of sunlight on milk flavor.

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TENDEROMETER READINGS AS AN INDEX OF QUALITY OF FRESH ASPARAGUS[1] A PRELIMINARY PAPER

R. R. JENKINS AND F. A. LEE[2]
New York State Agricultural Experiment Station, Geneva, New York

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Much interest has been shown lately in obtaining an objective method for determining tenderness or maturity of fresh vegetables which will serve as an index for establishing definite grades and standards for buying from growers and for predicting the quality of the finished product. Among the factors that are taken into consideration in arriving at an estimate of the quality grades of canned asparagus are color, uniformity of size, absence of defects, tenderness, and texture. The relative importance of each component has been expressed numerically on a scale of 100. Of these the Agricultural Marketing Service has scored tenderness as 40 per cent of the total. Inasmuch as tenderness is given major importance on this scale as an index of quality, any attempt to place the grading of asparagus upon a physical or chemical basis will have for its most important problem that of the measurement of maturity, and consequently emphasis has been placed to this end.

Various workers cited by Working (1924), Chatfield and Adams (1931), and Culpepper and Moon (1935) have found that considerable variations occur in the composition of fresh asparagus. This may be attributed to differences in cultural treatments, seasonal conditions, and other uncontrolled factors; also to differences in age of the young shoots and in amounts of the basal portion included in the samples.

MacGillivray (1933) studied the maturity of asparagus with a fruit pressure tester at three different times during the cutting season. He found that there was a tendency for the stalks to become more

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resistant to the tester as the season advanced. The following year, however, the situation was reversed. The base of the spear was usually tougher than the tip but it was possible to find all sorts of individual variations. There was a slight increase in toughness as the number of days required to grow a seven-inch spear increased.

Bisson, Jones, and Robbins (1926) studied some of the factors influencing the quality of fresh asparagus after it was harvested. They used the percentage of crude fiber as an indication of the toughness of asparagus. Their data show that at a temperature of 21.1 to 26.7°C.(70 to 80°F.), with the butts in water, the percentage of crude fiber in the dry matter changed from 10.54 to 15.51 in 72 hours, whereas at a temperature of 7.2 to 10°C.(45 to 50°F.) the change was from 10.54 to 12.71 during the same interval.

Culpepper and Moon (1935) found that pressure readings showed the greatest resistance at the base of the stalks because of fiber development. The quality of freshly cooked asparagus shoots four inches tall did not vary greatly as the cutting season advanced. This did not hold true, however, for shoots of greater height, owing to their tendency to become fibrous at the base and to branch and flower at a lower height late in the season. This made the taller stalks much less desirable late in the season than stalks of equal height cut earlier.

Recent applications of the tenderometer in forecasting the tenderness and quality of peas were used by Campbell (1939), Martin, Lueck, and Sallee (1938), Walls and Kemp (1939), Walls and Hunter (1938), and Lee (1939). In view of these findings it was deemed advisable to study the relative merits and limitations of the tenderometer in grading asparagus under practical conditions and to correlate these findings with crude-fiber analysis and organoleptic tests for quality ratings. This work was carried on during the past season at the Snider Packing Corporation, Mount Morris, New York. The tenderometer used in this work was essentially the same as that described in detail by Martin, *et al.* (1938). This model differed from his in that the grid was returned to the original position by a hand lever after shearing the asparagus.

EXPERIMENTAL PROCEDURE

Fresh samples of asparagus (Mary Washington variety) were taken at three different periods during the cutting season from three different types of soil—upland sandy, upland light clay, and river bottom—in order to obtain as wide a maturity spread as possible. Approximately 20 to 30 pounds of asparagus were taken from each type of soil plot, graded into three sizes—tiny, medium, and jumbo—and cut into four-inch lengths from the tip end of the spear, since

this length fits the sample chamber of the tenderometer. The chamber was then filled full by alternating the spears so as to insure uniformity, and a reading was taken (Table 1).

In addition, sample packs (13-ounce Birds Eye cartons) were prepared from each series of experiments by taking the unblanched graded asparagus cuts and freezing the contents immediately with "dry ice." The crude fiber content was then assayed as soon as possible on these samples by grinding the entire contents of the frozen package through a $\frac{3}{16}$-inch screen in a Sep-ro-siv at —18°C.(0°F.), mixing thoroughly, and taking 25 grams for analysis according to the A.O.A.C. (1935) method. Dry-matter determinations were also made on the fresh samples dried at 95°C.(203°F.) for 48 hours.

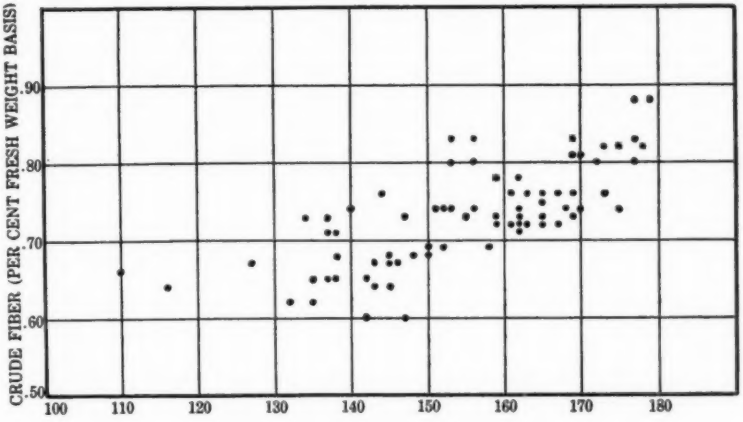

FIG. 1. Maturity studies on fresh asparagus.

Organoleptic tests were made on the cooked frozen samples by three food experts from the Frosted Foods Sales Corporation. These samples were previously prepared from each series of experiments by washing, blanching (scalding) in boiling water, cooling, packaging, and quick-freezing with "dry ice."

DISCUSSION OF RESULTS

The results of this study on the relation of tenderometer reading for the fresh asparagus to the crude fiber content and organoleptic tests are presented (Table 1, Fig. 1).

A very high correlation coefficient, 0.729 ± 0.029, is shown between the tenderometer readings and crude fiber content (Fig. 1).

TABLE 1

Maturity Studies on Fresh Asparagus

Treatment	Date sampled	Soil type	Size of spear	Tenderometer		Dry matter	Crude fiber	Organoleptic tests
				Stalks used	Pressure reading			
					lbs./sq. in.	*pct.*	*pct.*	
Freshly cut	5/18/39	River bottom	Tiny	23	140	9.86	.73	.....
Held overnight [1]	5/18/39	River bottom	Tiny	23	152	11.80	.....	.....
Freshly cut	5/18/39	River bottom	Medium	12	127	9.49	.68	.....
Held overnight [1]	5/18/39	River bottom	Medium	11	152	10.59	.....	.....
Freshly cut	5/18/39	River bottom	Jumbo	9	127	9.50	.67	Fancy
Freshly cut	5/19/39	River bottom	Tiny	15	129	9.79	.....	.....
Freshly cut	5/19/39	River bottom	Medium	11	138	9.76	.....	.....
Freshly cut	5/25/39	River bottom	Tiny	24	159	7.95	.74	Standard
Held overnight [1]	5/25/39	River bottom	Tiny	34	172	8.52	.....	.....
Freshly cut	5/25/39	River bottom	Medium	17	155	7.37	.69	Fancy
Held overnight [1]	5/25/39	River bottom	Medium	16	170	7.56	.....	.....
Held overnight [2]	5/25/39	River bottom	Medium	22	189	8.99	.....	.....
Freshly cut	5/25/39	River bottom	Jumbo	10	150	7.95	.68	Standard
Freshly cut	5/26/39	River bottom	Tiny	27	166	7.76	.76	Standard
Freshly cut	5/26/39	River bottom	Tiny	31	177	.....	.80	Standard
Freshly cut	5/26/39	River bottom	Medium	16	166	8.00	.72	Standard
Freshly cut	5/26/39	River bottom	Medium	15	165	.....	.75	Standard
Freshly cut	5/26/39	River bottom	Medium	14	170	7.48	.81	Standard
Freshly cut	5/26/39	River bottom	Jumbo	10	169	8.19	.73	Standard
Freshly cut	6/ 6/39	River bottom	Tiny	25	161	8.02	.73	Standard
Held overnight [2]	6/ 6/39	River bottom	Tiny	33	179	.....	.....	.....
Freshly cut	6/ 6/39	River bottom	Medium	15	161	7.29	.72	Standard
Held overnight [2]	6/ 6/39	River bottom	Medium	17	183	.....	.....	.....
Freshly cut	6/ 6/39	River bottom	Jumbo	8	162	7.00	.71	Standard
Freshly cut	6/ 7/39	River bottom	Tiny	29	151	7.51	.74	Standard
Freshly cut	6/ 7/39	River bottom	Medium	16	162	7.18	.72	Standard
Freshly cut	6/ 7/39	River bottom	Jumbo	10	147	6.91	.73	Standard
Freshly cut	5/25/39	Upland sandy soil	Tiny	17	132	.....	.....	.....
Freshly cut	5/25/39	Upland sandy soil	Medium	17	145	7.10	.67	Fancy
				14	143	.....	.67	Fancy
Held overnight [1]	5/25/39	Upland sandy soil	Medium	17	156	7.86	.....	.....
Freshly cut	5/25/39	Upland sandy soil	Jumbo	10	144	7.74	.64	Fancy
Held overnight [1]	5/25/39	Upland sandy soil	Jumbo	11	162	8.38	.....	.....
Freshly cut	5/26/39	Upland sandy soil	Tiny	30	169	7.30	.81	Standard
Freshly cut	5/26/39	Upland sandy soil	Tiny	36	177	8.47	.83	Standard
Freshly cut	5/26/39	Upland sandy soil	Medium	17	175	6.95	.74	Standard
Freshly cut	5/26/39	Upland sandy soil	Medium	20	165	8.37	.76	Standard
Freshly cut	5/26/39	Upland sandy soil	Medium	13	161	8.27	.....	.....
Freshly cut	5/26/39	Upland sandy soil	Jumbo	11	166	6.76	.76	.....
Freshly cut	5/26/39	Upland sandy soil	Jumbo	8	173	.....	.....	.....
Freshly cut	6/ 6/39	Upland sandy soil	Tiny	32	178	8.95	.88	Standard
Freshly cut	6/ 6/39	Upland sandy soil	Medium	13	175	8.00	.82	Standard
Freshly cut	6/ 6/39	Upland sandy soil	Jumbo	8	169	7.53	.74	Standard
Freshly cut	6/ 7/39	Upland sandy soil	Tiny	28	155	8.58	.83	Standard
Freshly cut	6/ 7/39	Upland sandy soil	Medium	16	162	8.07	.78	Standard
Freshly cut	6/ 7/39	Upland sandy soil	Jumbo	11	156	7.56	.80	Standard
Freshly cut	5/25/39	Upland light clay	Tiny	33	143	8.74	.68	Fancy
Freshly cut	5/25/39	Upland light clay	Tiny	32	145	8.24	.....	.....
Held overnight [1]	5/25/39	Upland light clay	Tiny	37	161	.....	.....	.....
Freshly cut	5/25/39	Upland light clay	Medium	16	140	.....	.65	Fancy
Held overnight	5/25/39	Upland light clay	Medium	19	160	7.58	.....	.....
Freshly cut	5/25/39	Upland light clay	Jumbo	12	137	7.06	.....	.....
Freshly cut	5/26/39	Upland light clay	Tiny	34	144	7.39	.67	Fancy
			Tiny	31	144	.....	.76	Standard
Freshly cut	5/26/39	Upland light clay	Medium	20	144	7.00	.60	Fancy
Freshly cut	5/26/39	Upland light clay	Medium	17	143	7.69	.....	.....
Freshly cut	6/ 6/39	Upland light clay	Tiny	31	136	7.18	.73	Standard
Freshly cut	6/ 6/39	Upland light clay	Medium	17	134	6.90	.62	Fancy
Freshly cut	6/ 7/39	Upland light clay	Tiny	33	138	7.62	.71	Standard
Held overnight	6/ 7/39	Upland light clay	Tiny	38	153	.....	.....	.....
Freshly cut	6/ 7/39	Upland light clay	Medium	18	136	6.83	.65	Standard

[1] Asparagus cuts held overnight on top of table at room temperature. [2] Asparagus cuts held overnight as above and then placed in direct sunlight the following day for six hours.

The lack of a perfect correlation may have been due to individual variations in composition of the stalks, as previously pointed out by MacGillivray (1933). Likewise, some of the asparagus may have been cut a few hours earlier than another lot. This holding period may have been sufficient to allow the asparagus to become somewhat tougher, especially if it happened to be a warm day. Freshly cut spears of asparagus, for example, gave a tenderometer reading of 155, but after holding overnight at room temperature gave a reading of 170, and when placed in direct sunlight for six hours a higher reading of 189 was obtained (Table 1).

A very high biserial correlation coefficient, -0.900 ± 0.054, was obtained between tenderometer and organoleptic tests. This indicates that the tenderometer has decided advantages in indicating the tenderness or quality of asparagus. Some advantages of the use of the tenderometer as an objective method are its simplicity of operation, rapidity, and the high degree of accuracy with which one may predict the quality of the raw stock. This would be of great value to growers, canners, and freezers.

In addition, the exceptionally high biserial correlation coefficient, -0.998 ± 0.035, was obtained between the percentage of crude fiber and the organoleptic tests. Although the crude-fiber content appears to be an excellent index of maturity, its estimation is comparatively tedious and time consuming and would not be practical as an objective method; however, the crude-fiber content would be of use for control work. As a tentative maturity standard, a fancy grade of asparagus would have a tenderometer reading of 150 or lower and any reading higher than this would be classed as "standard" from the standpoint of maturity only. A fancy grade based on maturity alone would also have a crude fiber content not exceeding .70 per cent or lower.

The coefficient of contingency between tenderometer and individual size of spears was found to be .175. This low factor would indicate that the measurement for tenderness by the tenderometer may be made either on the graded or ungraded samples depending on whether it is desired to evaluate the general quality of the asparagus as received at the plant or to determine the quality of the individual sizes. The tenderometer reading on the composite sample is therefore indicative, in a general way at least, of the tenderness of the asparagus present in the greatest proportion, the effect of the proportionality factor being determined by the magnitude of the difference in the tenderness of the various sizes composing the sample.

It is not always the case, apparently, that the tiny spears are tougher than the medium and jumbo of the same harvest, regardless of the statements of commercial packers. There are times, however, when this general conception may be correct.

SUMMARY

For a given variety of fresh asparagus grown under given conditions, the tenderometer was found to give a high correlation for tenderness and quality when compared with crude-fiber content and organoleptic tests. The tenderometer should serve, therefore, as a desirable objective method. In the light of preliminary data presented at this time, no definite limitations of tenderometer readings can be given for the different commercial grades of asparagus. The authors feel, however, that, as a tentative maturity standard, a tenderometer reading of 150 or lower indicates fancy-quality asparagus.

For grading purposes by means of the tenderometer, it appears unnecessary to separate the asparagus into the several commercial sizes.

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PECTINS AND THE TEXTURE OF COOKED POTATOES[1]

MONROE E. FREEMAN AND W. S. RITCHIE
Massachusetts Agricultural Experiment Station, Amherst, Massachusetts

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The degree of cell separation in mealy and waxy potatoes has been considered a significant and distinguishing character by many investigators. The observations of Coudon and Bussard (1897), Day (1909), Butler, Morrison, and Boll (1913), Sweetman (1936), and others have suggested that the solution of pectic materials caused the separation of the cells and thus determined the mealiness of the cooked potato.

Two reports have linked cell separation in cooked potato tissue with pectic materials by measuring changes in tensile strength. Davison and Willaman (1927) demonstrated by enzyme reactions that tensile strength (cell adhesion) of potato tissue depended on the pectic materials. Personius and Sharp (1938) showed that cooking markedly reduced the tensile strength; however, they found no consistent relation between the loss of tensile strength and mealiness.

Chemical analyses for pectin have given some additional data. It may be argued that if the degree of cell separation does cause mealiness, it is reasonable to suppose that some pectic fractions would be more difficult to extract from waxy tissue than from mealy tissue. The chemical analyses of several investigators, however, have not supported this hypothesis. Coudon and Bussard (1897) did not find that pectin content related to mealiness. Sweetman (1936) fractionated potato pectin by the method of Appleman and Conrad (1926) with similar results. Barmore (1937) used the Dastur and Agrihotri (1934) modification of Nanji and Norman's (1928) method and found no significant relation. These results appear to discount the hypothesis that cell separation is causally related to mealiness. Since experiments in this laboratory have indicated that Sweetman and Barmore may not have obtained fractions most likely to test this relationship, it seemed desirable to extract other pectin fractions from raw and cooked potatoes by methods specifically adapted to this purpose.

METHODS OF ANALYSIS

The extraction of total pectin by the methods of Nanji and Norman (1928) and Dastur and Agrihotri (1934) was tedious and uncer-

[1] Contribution No. 351, Massachusetts Agricultural Experiment Station, Amherst, Massachusetts.

tain when applied to potatoes. Elimination of large amounts of gelatinized starch and losses resulting from several manipulations were major difficulties. The methods developed by Appleman and Conrad (1926) and Conrad (1926) were rapid and reliable when modified to eliminate the starch. The method adopted for total pectin combined features of both methods.

Four extractions (four hours each) of 2.5 grams of dry-potato sample (or equivalent in fresh tissue) were made with 50 to 100 ml. of .5 per cent ammonium citrate at 85°C.(185°F.). Each extract was easily and sharply separated from the gelatinized residue by centrifuging in a Swedish angle centrifuge [2] at 4,000 r.p.m. A satisfactory separation could not be made by filtration or by an ordinary centrifuge. The combined extracts, almost water clear, were preserved with toluene and treated with ptyalin to hydrolyze the soluble and finely dispersed starch. The water-clear, starch-free filtrates were treated with NaOH, acetic acid, and $CaCl_2$ according to Branfoot's (1929) method for isolating calcium pectate. Because of the buffering action of ammonium citrate, precautions were taken that sufficient NaOH had been added to raise the pH to a value that would insure the hydrolysis of pectin to pectic acid. This was conveniently done by titrating (glass electrode) to about pH 11 or 12. The calcium pectate gel was filtered and washed with boiling water on tared filter paper, dried, and weighed in stoppered weighing bottles. The gels were clear and barely visible when suspended in water. All precipitates that were white or noticeably opaque were assumed to be contaminated with starch or protein and were discarded.

The acid-soluble fractions were extracted with .5 per cent oxalic or citric acid. The oxalic acid method, tedious and uncertain, was abandoned. The citric acid extracts were titrated to pH 7 (glass electrode) before treatment with ptyalin, then hydrolyzed, precipitated, and weighed as calcium pectate.

The water-soluble fractions were found to vary from 0 to 100 per cent of the total pectin, depending on the conditions of extraction. Consequently standardized procedures were designed to extract only a fraction of the water-soluble pectin. These procedures were as follows:

1. Extraction with cold water, according to Appleman and Conrad (1926), Conrad (1926), Dastur and Agrihotri (1934), and others, yielded only traces of calcium precipitate from fresh raw samples.

[2] If an efficient centrifuge is not available, the starch can be removed from crude extracts by freezing and thawing. However, part of the pectin cannot be easily washed from the spongy, fibrous, starch coagulum.

Apparently this precipitate was so contaminated that the presence of significant amounts of calcium pectate was questionable. When applied to air-dry samples, no calcium pectate could be obtained after a bulky protein coagulum had been removed by warming with a few drops of acetic acid.

2. When samples of cooked potatoes, air-dry or freshly prepared, were extracted with water at 37°C.(98.6°F.), they yielded about half of their total pectin to two 24-hour extractions. The time, temperature, size of sample, and volume of extracts were carefully standardized because this procedure extracts only part of the available pectin. According to the postulated relation between mealiness and pectin the percentage of total pectin extracted in this manner should be a measure of the extent of protopectin degradation brought about by cooking. Thus mealy potatoes should yield a greater portion of their total pectin than waxy samples.

3. Extractions were made with water at 85°C.(185°F.) according to Nanji and Norman (1928). Applied to cooked potatoes, this procedure gave in some cases almost all of the total pectin and in every case as much as the acid extraction. Applied to uncooked potatoes, this procedure yielded as much pectin as the acid extraction but only a portion of the total. Consequently the raw samples were extracted twice (four-hour periods) with water at 85°C. The conditions of time, temperature, sample, and volume of extract were again carefully standardized.

All results are reported as milligrams of calcium pectate per gram of dry sample or as percentage of the total pectin. Each value is the average of at least three (often six or more) determinations. The average variation in a set of check determinations was $\pm.5$ mg., the maximum variation being rarely more than ±1 mg. The values reported in percentage are accurate within ±2.5 per cent.

STORAGE OF POTATO SAMPLES

Four varieties were stored at low temperatures, 1.7 to 4.4°C.(35 to 40°F.) to retard pectin losses reported by Dastur and Agrihotri (1934). No loss occurred in six months at this temperature (Table 1).

Two uncooked samples, Golden and Idaho Russet, had lower values at the two-month storage period. This was attributed, however, to a sample drying process that allowed greater opportunity for enzymatic destruction of pectin. In most cases there was a small loss of pectin owing to baking.

PREPARATION OF SAMPLES FOR ANALYSIS

Conrad (1926) reported rapid enzymatic changes in pectin during preparation of samples. He recommended dipping potatoes in boiling

ethanol immediately after slicing. Steaming for one hour as well as hot-ethanol treatment preserved the largest amount of total pectin (Table 2). Baking and rapid air-drying involved small but significant losses.

While the hot ethanol adequately protected the total pectin, it was found that even this mild heat treatment was sufficient to mate-

TABLE 1

Total Pectin (Calculated as Ca Pectate) Content of Potatoes After Storage at 1.7 to 4.4°C.(35 to 40°F.)

Storage time	Raw				Baked			
	Bliss Triumph	Golden	Rural Russet	Idaho Russet	Bliss Triumph	Golden	Rural Russet	Idaho Russet
mo.								
2	.....	20.0	23.5	20.0	.....	20.0	23.0	20.0
4	23.0 [1]	26.5	24.5	23.4	20.0	20.5	22.2	21.5
6	23.5	26.8	26.5	23.5	19.5	22.5	22.5	20.8

[1] Mg. of calcium pectate per gram of dry sample.

rially shift the distribution of the pectin fractions. Data of Personius and Sharp (1938) also showed that chemical alteration of pectin began at temperatures considerably below cooking temperatures. The portion of the total pectin (in per cent) extracted at

TABLE 2

Effect of Sample Treatment on Yield of Total Pectin (Calculated as Ca Pectate)

Treatment of sample	Golden	Rural Russet	Idaho Russet
Dried raw	20.0 [1]	23.5	20.5
Hot ethanol dip	25.5	26.5	26.0
Steamed, dried	24.0	25.5	26.0
Baked, dried	20.0	25.0	20.0

[1] Mg. of calcium pectate per gram of dry sample.

37°C.(98.6°F.) from air-dried samples that had been sliced raw, treated with hot ethanol, and baked is recorded (Table 3).

Accordingly, dried samples of raw, steamed, and baked potatoes as well as fresh samples of raw and baked potatoes were prepared for analysis as follows:

1. About three to five kilos of raw potatoes were pared by hand, sliced by machine, and dried in shallow layers in glass trays over a steam radiator under electric fans. The slices were thoroughly dry in eight to 10 hours.

2. Steamed samples were cooked until done. The skins were removed and the hot flesh put through an ordinary potato ricer. The riced mass allowed a rapid circulation of warm air, and drying was complete in six to eight hours. Unless the cooked samples were riced, decomposition set in before samples were dry.

3. Baked samples were cooked in an ordinary oven at 218.3°C. (425°F.) until done—about one hour. These were riced and dried. All dried samples were ground in a Wiley mill, a ball mill, and screened. Samples passing the 50-mesh but not the 100-mesh screen and samples passing the 100-mesh but not the 200-mesh screen were retained.

Analyses were also made on fresh samples without drying. In this case the raw potatoes were finely ground, thoroughly mixed, and

TABLE 3

Yield of a Water-Soluble Pectin Fraction (Calculated as Ca Pectate) From Air-Dry Samples Baked, Dipped in Hot Ethanol, and Sliced Raw Before Drying

Treatment of sample	Golden stored 2 months	Rural Russet stored 2 months	Idaho Russet stored 2 months	Golden stored 4 months	Idaho Russet stored 4 months
Raw, dried	0[1]	0	0	0	0
Hot-ethanol dip, dried	17	19	17	16	10
Baked, dried	35	68	60	50	72

[1]Per cent of the total pectin calculated as Ca pectate. Experimental error not greater than ±2.5 per cent.

immediately weighed into flasks for analysis and weighing bottles for moisture determination. The cooked samples were ground with water to a thick paste convenient to pour and mix. The paste was immediately sampled for pectin and moisture analyses.

FRACTIONATION OF PECTIC MATERIAL

Nearly all methods that have been used for quantitative determination of pectic fractions are quite similar to either the method proposed by Nanji and Norman (1928) or that of Conrad (1926). These representative methods differ primarily in the estimation of the water-soluble pectin and the protopectin. Nanji and Norman extracted with water at 85°C.(185°F.), while Conrad defined water-soluble pectin as that exhaustively extracted with cold water. The results of these methods applied to potato samples are recorded (Table 4).

It is interesting to note that a series of short extractions with water at 85°C.(185°F.) removed as much pectin as the acid at 85°C. (Table 4). The lower acid values were probably due to some destruc-

tion of the pectin. Apparently acids were not necessary to render a large part of the pectic substance water soluble. If this material was protopectin, it was so loosely bonded to the cell-wall material that it was hydrolyzed completely in a few hours by water at a hydrogen-ion concentration near pH 7. Possibly the reaction was one of peptization by hot water rather than a chemical hydrolysis of the cellulose-pectin bonds postulated for protopectin.

The remainder of the material not extracted by acid or hot water but only by ammonium salts is usually designated calcium or magnesium pectate and pectic acid. Why a small but significant part of this fraction was rendered water soluble by heating the potato tissue cannot be easily explained.

TABLE 4

Yields of Pectin Fractions by Ordinary Methods of Extraction (Calculated as Ca Pectate)

Variety	Treatment	1 Extracted by ammonium citrate	2 Extracted by citric acid	3 Extracted by water at 85°C. (185°F.)	4 Extracted by water at 37°C. (98.6°F.)	5 Pectates column 1-3
Golden	Raw dry	20.0[1]	2.5	5.5	0.0	14.5
Rural Russet	Raw dry	23.5	7.5	10.5	0.0	13.5
Idaho Russet	Raw dry	20.0	12.5	12.5	0.0	7.5
Golden	Ethanol	25.5	14.0	14.5	4.0	11.0
Rural Russet	Ethanol	26.5	16.5	23.5	5.0	3.0
Idaho Russet	Ethanol	25.5	14.6	15.0	4.5	10.5
Golden	Steamed	24.0	11.0	18.5	10.0	5.5
Rural Russet	Steamed	25.5	16.3	16.4	13.5	9.1
Idaho Russet	Steamed	26.0	18.0	17.0	14.0	9.0
Bliss Triumph	Baked	20.0	.....	13.5	14.5	6.5
Golden	Baked	20.0	11.0	15.5	7.0	4.5
Rural Russet	Baked	25.0	16.5	16.0	17.0	9.0
Idaho Russet	Baked	20.0	15.0	19.0	14.5	1.0

[1] Mg. of calcium pectate per gram of dry sample.

At any rate only two pectin fractions were clearly indicated here. A fraction was removed by hot water and became increasingly soluble in cold water as the tissue was heated. A second fraction was removed only by ammonium salts. Since the former fraction was readily altered by heat, this fraction was partially extracted from raw and cooked potatoes to see if the ease of extraction was related to mealiness. The three raw samples in Column 3 (Table 4) supported this idea since the larger amount of pectin was obtained from the mealy samples. This seemed to indicate a greater degradation of the intercellular binding material. Since the results with the other

samples did not favor this idea, further tests were made (Tables 5 and 6).

Analyses of 10 different samples made after two months' storage and four samples after four months' and six months' storage are recorded (Table 5). All samples were air-dried before analysis. Raw samples were extracted for two periods (four hours each) with 70 ml. of water at 85°C. The baked samples were extracted twice (24

TABLE 5

Total Pectin (Calculated as Ca Pectate) Extracted at 37 and 85°C. (98.6 and 185°F.) From Dried Samples of Baked and Raw Potatoes

Variety	Storage time	Texture of baked potato [1]	Baked samples	Raw samples
			Extracted at 37°C.	Extracted at 85°C.
	mo.			
Golden	2	1.0	33 [2]	11 [2]
Rural Russet	2	3.5	60	26
Idaho Russet	2	4.0	73	35
Bliss Triumph	4	1.0	74	40
Golden	4	1.0	48	30
Rural Russet	4	3.0	80	34
Idaho Russet	4	4.0	70	30
Bliss Triumph	6	1.0	86	40
Golden	6	1.0	60	34
Rural Russet	6	3.0	75	51
Idaho Russet	6	3.5	62	35
Bliss Triumph II [3]	2 (or less)	1.0	65	32
Golden II	2 (or less)	1.0	57	30
Rural Russet II	2 (or less)	2.0	80	43
Green Mt. II	2 (or less)	2.0	90	86
Russet Burbank II	2 (or less)	2.5	51	36
Idaho Russet II	2 (or less)	4.0	54	84
Green Mt. II	2 (or less)	4.0	62	55

[1] Texture was estimated according to Sweetman (1936). [2] Per cent of the total pectin (calculated as Ca pectate). [3] Roman numerals refer to a second set of samples collected in 1937.

hours each) with 100 ml. of water at 37°C. The results are reported as percentage of the total pectin.

Analyses were also made on fresh tissue from 14 additional samples of potatoes [3] (Table 6).

The data do not show any consistent relation between the quantity of the pectin fractions isolated and mealiness rating (Tables 5 and 6). From this it seems apparent that mealy potatoes either

[3] These samples were furnished by Dr. Chucka of the Maine Agricultural Experiment Station. The authors wish to express their appreciation of this assistance.

cooked or raw did not yield any more water-soluble pectin than did the waxy samples.

From the analyses in this report and the data obtained by Personius and Sharp (1938) it seems likely that the degradation of potato pectin to easily soluble forms has taken place during the initial period of the cooking process before the tissue has reached a condition that can be described as mealy or waxy.

TABLE 6

Total Pectin and Fractions of Total Pectin (Calculated as Ca Pectate) Extracted by Water at 37 and 85°C.(98.6 and 185°F.) From Fresh Samples of Baked and Raw Potatoes

Sample	Texture of baked potato	Raw samples			Baked samples		
		Total pectin as Ca pectate	Extracted at 85°C.		Total pectin as Ca pectate	Extracted at 37°C.	
			Pectin as Ca pectate	Per cent of total pectin as Ca pectate		Pectin as Ca pectate	Per cent of total pectin as Ca pectate
Idaho Russet [1]	4 [2]	25.5 [3]	15.5 [3]	60 [4]	25.0 [3]	10.5 [3]	42 [4]
Green Mt. No. 213	4	18.5	11.4	62	18.5	11.5	62
Green Mt. No. 217	4	24.0	13.5	56	18.5	15.2	82
Green Mt. No. 231	4	19.5	14.5	75	19.0	13.5	71
Green Mt. (Mass.)	4	.....	13.5	....	.....	9.5	....
Green Mt. No. 235	2.5	26.5	.....	....	23.5	13.0	55
Green Mt. (Maine)	2	25.5	16.5	65	19.5	15.0	77
Cobbler	2	27.0	9.0	33	21.5	11.9	55
Green Mt. No. 216	2	25.0	7.5	30	18.5	10.5	57
Green Mt. No. 215	2	22.0	7.5	34	19.5	11.0	57
Green Mt. No. 214	2	26.0	8.5	33	21.5	10.0	46
Chippewa	2	28.0	16.0	57	20.5	15.0	73
Sebego	1	27.0	15.5	57	30.5	14.0	47
Bliss Triumph	1	32.5	6.5	20	29.0	13.0	45

[1] These samples were collected in 1938; all but two were supplied by Dr. Chucka of the Maine Agricultural Experiment Station. [2] Texture was estimated according to Sweetman. [3] Mg. of Ca pectate per gram of dry sample. [4] Per cent of the total pectin (calculated as Ca pectate).

SUMMARY

Minimum changes in potato-pectin fractions occurred when sliced tissue was rapidly dried in air. Heat treatment barely sufficient to inactivate the enzymes rendered a part of the pectin soluble in water.

Hot-acid extraction of potato tissue did not define the so-called protopectin fraction. Water under the same conditions extracted as much if not more pectin.

It is suggested that potato pectin can be adequately defined by two fractions:

1. A fraction that is soluble in ammonium citrate (or oxalate) and insoluble in hot water. This fraction may be pectic acid or insoluble salts of the acid.

2. A fraction that is easily dispersed by hot water but not by cold water. Since this fraction is so easily removed, it is possible that it is not the postulated cellulose-pectin complex of protopectin but rather a lyophilic gel that is readily peptized by warm water.

Analyses of water-soluble pectin fractions of raw and cooked potatoes offer additional evidence that the solution or degradation of pectic material does not determine mealiness in potatoes.

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COMPARISON OF NUTRITIVE VALUE OF REFINED COCONUT OIL AND BUTTERFAT[1]

ROBERT S. HARRIS AND L. MALCOLM MOSHER
Biological Research Laboratories, Massachusetts Institute of Technology, Cambridge, Massachusetts

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This study was undertaken to determine whether there is a difference in the general nutritive value of butterfat and coconut oil and whether diets containing 25 per cent of either of these oils will produce fatty infiltration or degeneration of the liver when fed to young, healthy, adult rats.

No reports were found in the scientific or clinical literature which indicated that there is a significant difference in the nutritive value of these fats or which indicated that they were toxic when taken in ordinary diets in normal quantities by normal individuals, in normal quantities by abnormal individuals, or in abnormal quantities by normal individuals. It has been established that the animal body has the ability to synthesize its fats from the food fats or other compounds which occur in the food.

Significant portions of mankind consume large quantities of coconut oil. Some tropical peoples use coconut oil exclusively in cooking.

Fatty degeneration of the liver can be produced by an abnormal diet in which certain amino-acid constituents (gliadin), according to Tucker and Eckstein (1938), are low or certain others (methionine) are present in increased quantities. Such fatty livers are only of clinical interest, for these diets are entirely artificial and the development of fatty livers is not dependent upon the type of fat which is fed.

In the work being reported experimental animals were given diets which were abnormal in only one respect, in that they contained about four times the normal amount of fat. This was done to accentuate any tendency toward the production of abnormalities by the coconut oil or butterfat. In all other important respects they were well balanced and adequate for the support of active growth and normal health.

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[1] Contribution No. 167 from the Biological Research Laboratories, Massachusetts Institute of Technology, Cambridge, Massachusetts.

EXPERIMENTAL DIET

The experimental diets used in these tests were constituted as follows:

	Coconut-oil diet	Butterfat diet
Extracted milk powder	72%	72%
Butterfat	.....	25%
Coconut oil	25%	.....
Extracted yeast	3%	3%

Roller-dried skim-milk powder was extracted twice for 24 hours with ethyl alcohol and twice for 24 hours with anhydrous ethyl ether. The brewer's yeast was extracted by the same procedure. In addition, each animal received daily an equivalent of six U.S.P. units of vitamin D in the form of Viosterol (Mead's), an equivalent of 60 U.S.P. units of vitamin A (Hickman distillate, 200,000 units per gram), and an equivalent of three milligrams of iron in the form of ferric chloride. The vitamin A and vitamin D were fed in mineral oil and the ferric chloride was mixed in the diet.

The butterfat used in these studies was purchased as butter in three local retail stores and prepared by melting and passing through filter paper in a steam-jacketed funnel.

Five one-gallon cans of refined edible 76 coconut oil were received from Durkee Famous Foods, Chicago, Illinois. It was stated by the refiners to have the following characteristics, of which the free fatty-acid content, the iodine and saponification number, and the melting point, were confirmed by our analyses.

Color, Lovibond scale	0.7 red 5.0 yellow
Free fatty acid, calculated as oleic	0.014%
Refractive index, butyro at 48° C	30.9
Setting point	23.0°C.
Iodine value	8.85
Saponification number	256.5
Capillary melting point	77.0°C.
Flavor	Good

This oil was melted and passed through filter paper in a steam-jacketed funnel in the same manner as the butterfat.

STOCK DIET

An acceptable stock diet was fed to a third series of animals. This diet according to Russell (1932), consisted of 60 per cent ground whole wheat, 30 per cent dried whole milk, and 10 per cent meat scrap (Swift's) with the addition of sodium chloride to the extent

of two per cent of the weight of the wheat. The animals on this normal and adequate diet served as controls or standards of normality since they were litter mates to the animals used in the two experimental series.

EXPERIMENTAL PROCEDURE

Wistar strain albino rats were used in this investigation. They were placed on the experimental diets when 120 days old. Each animal was separately housed in a galvanized wire cage with raised bottom. Twelve males and 13 females were fed the coconut-oil diet, 12 males and 13 females were fed the butterfat diet, and four males and four females were fed the stock diet.

The diet was fed *ad libitum*, but the amount of food consumed by each rat was determined periodically. Distilled water was supplied at all times.

At the end of 15 days, three male and three female rats from each group were guillotined and sections of the liver, heart, lungs, kidneys, skin, subcutaneous fat, adrenals, and femur of each were taken for microscopic examination and the remaining portions of the internal organs were preserved in alcohol. The livers and the carcasses were submitted to the chemical analysis detailed below.

Similarly, at the end of 30, 60, and 90 days animals from each group were killed and studied in the same manner as those destroyed at the end of 15 days. In addition, at the end of 60 and 90 days rats (three and five, respectively) which had been on the stock diet were destroyed and submitted to the same pathological and chemical analysis.

Chemical Study of Body Tissue: The entrail-free carcasses of each group of animals were split into strips, passed through a meat grinder, and weighed. This material was then dried to constant weight at 105°C.(221°F.), pulverized in a coffee mill, placed in a Soxhlet extractor, and extracted for 18 hours with 95 per cent ethyl alcohol, then 18 hours with ethyl ether (U.S.P.). The solvent was removed from the combined extracts and the weight of the fat determined. From the data so obtained the moisture content and the fat content (alcohol-ether soluble fraction), of the body tissues were calculated.

Chemical Study of Liver Tissue: After a section of the liver of each animal was taken for pathological study the livers of each group were pooled, macerated, transferred to a watch crystal tared with one 11.0 centimeter ashless filter paper, and weighed. The samples were then dried to constant weight at 105°C., transferred quantitatively to Soxhlet extractors, and extracted for 18 hours with 95 per cent ethyl alcohol, then for 18 hours with ethyl ether (U.S.P.). The combined extracts were evaporated to dryness and weighed.

From the data the moisture content and the fat content (alcohol-ether soluble fraction) of the livers were calculated.

Determination of True Lipids in Liver and Body Tissues: The true lipid (petroleum-ether soluble) content of the liver and body tissues of the rats which had been on the three diets for 60 and 90 days was determined on the substance obtained by extracting the alcohol and ether extracts with petroleum ether.

EXPERIMENTAL RESULTS

Weight Increase: Growth of the animals during the test is shown by the following summary tabulation:

Days on diet	Body weight in grams 0	15	30	60	90	Total increase
Rats (25) on butterfat diet	208	217	229	228	240	32
Rats (25) on coconut-oil diet	208	217	232	244	258	50
Rats (8) on stock diet	210	224	235	246	274	64

These results indicate that during the first 30 days of the test, both groups of animals grew at the same rate, while during the next 60 days the animals on the coconut-oil diet grew much more rapidly than those on the butterfat diet. This increase in weight was due to an increase in body tissue, not adipose tissue, for subsequent data will show that the body and liver tissue of the group fed the coconut-oil diet was no higher in fat or lipid content than that of the group fed the butterfat diet.

Food Consumption: The average amount of food consumed each day by the animals during the test period is shown by the following tabulation:

Group	Diet	Average weight of food consumed daily by each animal during 15 days	30 days	60 days	90 days
1	Butterfat	8.0 gms.	11.8 gms.	10.9 gms.	11.3 gms.
2	Coconut oil	7.8 gms.	11.0 gms.	10.7 gms.	11.1 gms.

The data indicate that throughout the test the animals fed the butterfat diet consumed a slightly larger amount of diet. The superior weight increase of the group fed the coconut oil was not due to greater food consumption.

Results of Chemical Analysis of Body Tissues: Both the butterfat diet and the coconut-oil diet have a tendency to increase the fat (alcohol and ether-soluble fraction) content of the body tissues, the increase being essentially the same in both groups (Table 1).

The petroleum-ether soluble fraction (true lipid) of the body-tissue lipids was essentially the same at the end of 90 days for both

experimental groups and was about 25 per cent higher than that of the group fed the stock ration.

These chemical results indicate that a diet containing 25 per cent butterfat or 25 per cent coconut oil produces a slight fatty infiltration of the body tissues of rats which is measurable by chemical analysis.

TABLE 1

Summary of Chemical Data

Group	Diet	Days on diet	Moisture	Fat [1]		True lipid [2]
				Wet	Dry	
			pct.	*pct.*	*pct.*	*pct.*
Body Tissues						
1	Butterfat	15	65.9	10.3	30.7	.....
2	Coconut oil	15	67.9	9.3	29.7	.....
1	Butterfat	30	65.9	11.7	34.2	.....
2	Coconut oil	30	67.2	11.6	35.2	.....
1	Butterfat	60	64.3	12.5	34.9	24.0
2	Coconut oil	60	64.1	13.3	36.9	24.8
3	Stock	60	67.1	8.4	25.8	19.7
1	Butterfat	90	64.7	13.9	39.3	28.2
2	Coconut oil	90	64.9	13.7	38.8	27.4
3	Stock	90	61.9	11.3	29.6	23.2
Liver Tissues						
1	Butterfat	15	72.0	6.0	21.3	.....
2	Coconut oil	15	73.0	5.7	21.1	.....
1	Butterfat	30	71.6	6.5	22.3	.....
2	Coconut oil	30	71.5	6.2	21.7	.....
1	Butterfat	60	70.6	6.9	23.7	18.8
2	Coconut oil	60	70.4	7.2	24.0	18.5
3	Stock	60	70.8	4.8	16.5	13.6
1	Butterfat	90	70.5	7.3	24.6	19.3
2	Coconut oil	90	69.9	8.0	26.4	20.4
3	Stock	90	70.6	6.8	23.0	17.0

[1] Fat = alcohol and ether extract. [2] True lipid = petroleum-ether soluble lipid.

Results of Chemical Analysis of Liver Tissues: The data indicate that the fat content of the livers of animals on the butterfat and the coconut-oil diets progressively increased during the test period (Table 1). This tendency was essentially the same in both groups of animals.

The true lipid content of the liver tissue of both groups of experimental animals was essentially the same and 20 to 30 per cent higher than the true lipid content of the liver tissue of the group fed the stock ration.

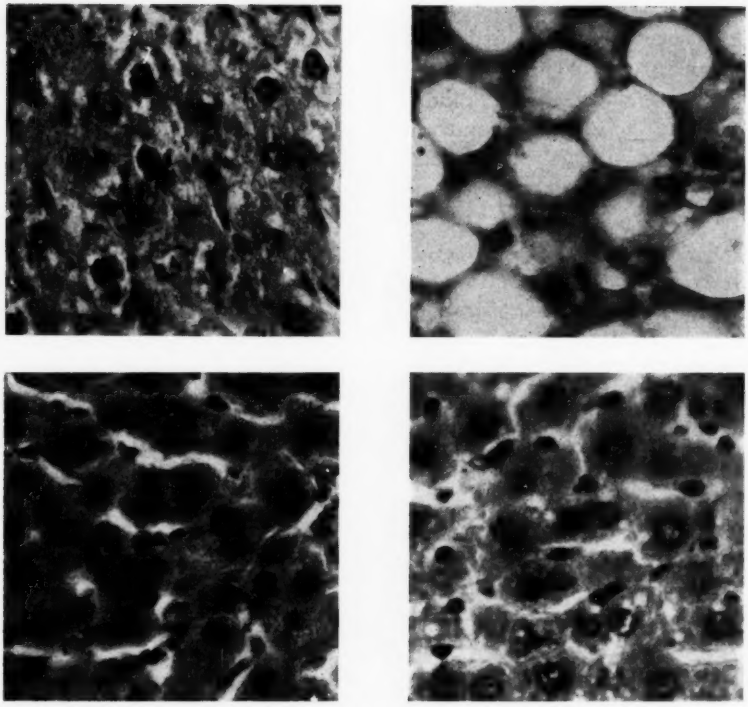

FIG. 1. Photomicrographs (500x) of typical liver sections stained with hemotoxylin and eosin. White vacuoles represent fat droplets.
Upper left: from rat fed stock diet.
Upper right: example of true fatty infiltration, animal not in this series of experiments.
Lower left: from rat fed coconut-oil diet.
Lower right: from rat fed butterfat diet.

Milk-powder diets, containing 25 per cent coconut oil or 25 per cent butterfat, therefore produce a measurable amount of fatty infiltration of the liver tissue of rats.

PATHOLOGICAL FINDINGS

The livers of all animals were studied histologically using Scharlach R and hemotoxylin and eosin stains. There was no significant variation within groups, between groups, or between series, nor was there any evidence of pathological fatty infiltration in any animal. There was evidence of a very slight amount of infiltration of fat into the cytoplasm of the liver tissues (Fig. 1).

There were no pathological findings on histologic examination of the lung, heart, kidney, spleen, gastrointestinal tract, skin, muscle,

subcutaneous tissue, and bone (femur) of any of the animals which had been on the experimental or stock diets for 90 days. Thus, histologically, the chemical observation that these diets produced a mild tissue infiltration was confirmed. Furthermore, these results indicate that coconut oil and butterfat are equally harmless to rats, even when they constitute rather high proportions of the diet.

SUMMARY AND CONCLUSIONS

Experimental rats were maintained on a diet consisting of extracted skim-milk powder (72 per cent), extracted brewer's yeast (three per cent), and refined coconut oil (25 per cent) or butterfat (25 per cent), supplemented with vitamin A, vitamin D, and iron. These diets were abnormal in only one respect, that is, they contained an abnormally large proportion of fat. The animals were observed as to the effects of these two diets on weight increase and food consumption. Groups were guillotined after 15, 30, 60, and 90 days on the diets, and body tissues were studied histologically. Results were compared with those on rats maintained on a standard stock ration which served as control.

Animals on the butterfat diet consumed a slightly larger, but possibly insignificant, amount of diet but increased in weight much less rapidly than the animals on the coconut-oil diet.

The superior weight increase of the rats on the coconut-oil diet was not adipose tissue, for the body and liver tissues of the groups contained essentially the same amount of fat (alcohol-ether extract) and true lipid (petroleum-ether extract of the alcohol-ether extract). The investigation is being extended to determine whether the weight increase was due to increase in muscle tissue.

The animals on both the butterfat and the coconut-oil diets developed a slight fatty infiltration of the body and liver cytoplasm. This was shown by increased amounts of fat and of true lipids and by histological examination. This fatty infiltration was equally intense in the coconut-oil and butterfat groups.

There was no evidence of pathological tissue changes in any animal in any group.

These results indicate that butterfat and coconut oil, even when fed at rather high levels in a complete diet, are equally harmless to rats and presumably to man.

ACKNOWLEDGMENT

Dr. Sidney Farber, pathologist, Infants' and Children's Hospital, Boston, prepared, examined, and interpreted all of the histological sections. We greatly appreciate his coöperation.

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WHEY SOLIDS IN CANDY[1]

B. H. WEBB AND C. F. HUFNAGEL
Division of Dairy Research Laboratories, Bureau of Dairy Industry, U. S. Department of Agriculture, Washington, D. C.

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Milk is produced primarily for human food; yet in the manufacture of cheese the whey, representing half of the milk solids, is generally discarded or fed to pigs. The investigation reported in this paper was undertaken in the hope that the results obtained would lead to an important outlet for whey solids in the candy industry. Candy is largely composed of dextrose, levulose, and sucrose, while whey solids contain 75 per cent lactose. It appeared reasonable to suppose that the carbohydrate of milk could be added to the list of sugars suitable for large-scale use in candy manufacture. The work was directed toward the development of whey-candy formulas which would utilize all the ingredients of whey and thus make unnecessary the costly separation of individual whey constituents.

PREPARATION OF WHEY SOLIDS FOR USE IN CANDY

When whey is separated from milk curd in the cheese-making process it contains approximately 6.4 per cent of whey solids. Fresh whey and various forms of concentrated whey were used in the making of candy. The use of sweet rennet-cheese whey, such as that derived from the manufacture of Cheddar and Swiss cheese, as well as the more acid cottage-cheese whey, was investigated.

Fresh, sweet, cheese whey was made into candy by drawing the ingredients to be used directly into the vacuum pan and condensing the mixture. The cooking was completed either in the vacuum pan or the batch was removed to the candy kettle and the boiling finished under atmospheric pressure. While some very satisfactory caramels were produced by this method, it would be practical only where vacuum-evaporating equipment was available. When fresh whey was used it was necessary to boil off approximately 1,400 pounds of water to obtain 100 pounds of whey solids. The economical removal of so much water requires either a vacuum pan or a spray-drying operation.

A soluble, spray-dried, sweet-whey powder was found satisfactory for use in candy; it could undergo long storage periods, was light

[1] A preliminary paper on this subject was presented by the senior author before the American Dairy Science Association, June, 1938. Abstract, J. Dairy Sci. 21, 156 (1938).

to transport, easy to reconstitute, and made excellent candy. Low-grade, insoluble powders were often excessively high in acid, difficult to disperse in the candy mixture, and generally ill suited for this purpose.

Plain condensed whey was an excellent source of whey solids in candy. Sweet, separated, Cheddar- or Swiss-cheese whey was pasteurized and condensed under vacuum to a solids content of 68 to 70 per cent. This represented the highest concentration of whey that was fluid enough to be drawn easily from the pan. The approximate concentration was determined by means of a striking cup and a balance. The specific gravity of the finished whey was about 1.35. The heavy viscous concentrate provided a suitable medium for the development of minute lactose crystals without special treatment. This was a very desirable circumstance, since small crystals were easy to dissolve in cooking the candy and the fine crystals contributed toward the production of a smooth-bodied candy when the concentrate was used without cooking. Plain condensed whey kept well under refrigeration for several weeks and, except for its perishable nature, it was a simple, economical, and satisfactory source of whey solids for candy.

A plain condensed product was made from acid cottage-cheese whey, the cheese having been made with rennet and starter. The whey, which had a titratable acidity of about .55 per cent after it was separated from the curd, was pasteurized and condensed in a stainless-steel vacuum pan. The concentrated product had a reaction of about pH 3.8 to 4.0, and this was sufficiently acid to keep it in good condition for many weeks when stored in air-tight containers in a cool place. Before the whey was used in candy the acid was neutralized with sodium bicarbonate to pH 5.6 to 5.8, the whey was warmed, and the CO_2 allowed to escape. When strong sodium hydroxide was used as a neutralizer it was difficult to prevent local action on the protein. Satisfactory candy was made with neutralized cottage-cheese whey but its flavor was often inferior to that made with sweet, cheese whey.

Sweetened condensed whey, according to Ramsdell and Webb (1938), was found to be the best whey product for candy manufacture. It was made as follows:

A quantity of sugar equal to the weight of the whey solids in the batch was added to separated and pasteurized fresh, sweet, rennet-cheese whey. The mixture was evaporated under vacuum to about 38 to 39° Baumé determined at pan temperature, or 79 to 80 per cent total solids. It was cooled to 35°C.(95°F.), seeded, stirred to crystallize the lactose, placed in air-tight containers, and stored, pref-

erably in a cool place. At room temperature it kept for months, but a gradual increase in viscosity and a darkening in color were observed. These changes did not affect its value in candy manufacture.

Sweetened condensed whey seems to be an excellent product for the small candy maker. It is simple and cheap to prepare, keeps well, and is easy and convenient to use. The large candy manufacturer who buys sugar at a lower cost than the small creamery operator and who can arrange for a constant whey supply might prefer plain condensed whey. Sweetened condensed whey was used as a source of whey solids in most of the work reported in this paper.

The stable whip produced by fat-free, sweetened, condensed whey offered an opportunity to use this material as a frappé and for the development of new types of candy. Its volume more than doubled after it was whipped for three to five minutes, and the whip remained stable for many hours.

Crude lactose was used in candy but its cost was prohibitive and it lacked some of the advantages of whey solids.

DEVELOPMENT OF CANDY FORMULAS

Many different candy-making procedures were investigated during the development of the routine methods which were finally used in making various types of candy. Cooking was done in a five-gallon stainless-steel candy kettle equipped with a double-action stirrer which revolved at 60 revolutions per minute. Such a mechanical stirring device was found to be very desirable in the cooking of whey-candy mixtures. One hundred pounds steam pressure was available in the kettle jacket, and provision was also made to circulate water through the jacket for cooling purposes.

Each batch of candy contained 5,000 grams (11 pounds) of solids. The ingredients were carefully weighed, transferred to the candy kettle, and cooked with stirring to the proper temperature. Upon completion of the 10- to 15-minute cooking period the batch was cooled and poured in accordance with the requirements of the type of candy being made.

More than 400 batches of candy were made in testing different forms of whey and in developing formulas for various types of candy. The formulas presented are based on the use of sweetened condensed whey, but other forms of whey solids can be used by making proper substitutions.

The general principles of candy making as practiced by the trade, Berman (1925), were adhered to for the standard types of candy. The formulas were developed experimentally on a small scale, and

some changes may be necessary in adapting them to factory production.

The use of whey solids necessitated making certain adjustments in the candy-making process. Since whey products contained varying amounts of lactic acid which tended to invert more than the usual amount of sucrose during cooking, it was necessary to consider this point when deciding upon the quantity of "doctor" or corn and invert sirup to use. High whey-solids candy required less "doctor" than did non-whey candy. Sweetened condensed and plain condensed whey made from sweet Cheddar or Swiss cheese varied in reaction between pH 5.3 and pH 5.9, while condensed or dry whey in which acid was allowed to develop before processing had a lower pH and caused more inversion of sucrose than a sweeter whey.

Coconut fat was used in the making of fudge, caramel, and taffy. From the standpoint of flavor, quality, and nutritional value, however, the use of butter or cream as a source of fat is highly advantageous. Milk fat may be substituted in whole or in part for coconut fat, but when a hard fat is replaced by a soft fat, such as butterfat, adjustment should be made in the cooking temperature.

The data (Table 1) concern a brittle type of candy or wafer which had a hard porous structure. Air whipped into sweetened condensed whey produced the characteristic texture of this candy. No gas-producing material was necessary to form the light porous body; the mixture was dried, not cooked. The dry precooked cereal was used to cover up the salty whey taste. Since lactose and sucrose were present in "Wheyfers" in an amorphous condition, the candy was coated with chocolate at once to prevent absorption of moisture and subsequent crystallization of lactose.

A series of fudge formulas which contain from 21 to 37 per cent whey solids are presented (Table 2). Experimental work was conducted to determine the best method for obtaining a fine, smooth, crystalline structure in whey fudge. When sugar fondant was added to the cooked batch in accordance with commercial practice, the fudge was slow to crystallize, and a coarse weak body was produced. When sweetened condensed whey or powdered lactose was added with the sugar fondant, an excellent, smooth, crystalline structure was obtained. Five grams of lactose was used in Formulas 1 and 2 (Table 2), while sweetened condensed whey was added as seed to fudge No. 3. The addition of sweetened condensed whey with the sugar fondant gave excellent results, but if more than two or three per cent of the weight of the batch was used, it was necessary to raise the cooking temperature slightly to compensate for the diluting effect of the water in the whey.

The optimum whey-solids content for fudge was between 20 and 30 per cent. Within this range a good milk-flavored candy was obtained; beyond 30 per cent whey solids a salty and sharp aftertaste was noticeable. In concentrations below 20 per cent whey solids the flavor of fudge was improved as the whey content was raised.

The formula for whipped fudge (Table 3) yielded a soft lightweight candy. The sugar and sirups, which were the only cooked constituents, did not require stirring while boiling. This fudge had a soft smooth structure, but it hardened and lost moisture quite readily unless its freshness was preserved by chocolate coating. The sweetened condensed whey used in whipped fudge contained very small lactose crystals. This was necessary to avoid a sandy coarse-grained product. The whey acted both as a fondant introducing sugar crystals and as a frappé introducing air.

The lactose as well as the sucrose in caramel and taffy (Tables 4 and 5) must be held in solution as a concentrated sirup to prevent the development of a short-bodied candy. This was accomplished by avoiding seeding, by making candy low in moisture, and by providing a high concentration of noncrystallizing substances.

Many batches of caramel were made in which the milk solids were completely replaced by whey solids. Such a candy was satisfactory in flavor but it possessed a weak sticky body and often grained quickly. Whey did not contain enough protein to replace skim milk as a builder of body in caramel, nor did whey protein appear as effective as casein in this respect. The caramel formula (Table 4) contained the minimum quantity of skim-milk solids needed to produce a firm-bodied candy. Materials such as starch or flour, which are sometimes utilized for this purpose, were not used in this work. To a limited extent, high-whey, low-milk caramel was improved in body by using large quantities of hard coconut fat. Firm-bodied whey caramels, well fortified with corn and invert sirups, retained a chewy non-grainy body when the whey-solids content was held below 28 to 30 per cent.

The formula for a whey taffy (Table 5) contains only sweetened condensed whey, corn sirup, and such flavoring as vanilla, peppermint, or maple. The taffy was improved by the addition of chocolate, butterfat, or coconut fat. Such materials were added in amounts ranging from five to 10 per cent and were substituted for sweetened condensed whey solids; for example, chocolate taffy contained the following:

	Grams	Per cent solids
Corn sirup	2,500	40
Sweetened condensed whey	3,250	52
Chocolate	400	8

The taffy (Table 5) was slow to grain, but an increase in sweetened condensed whey and a decrease in corn sirup promptly produced an unstable body. Very soft whey taffy quickly developed a granular body during storage.

Other kinds of candy were made with whey solids, among which were several candy centers to be used for chocolate coating. It was not possible to prepare a pure white fondant containing whey because interaction between the carbohydrates and whey protein caused a fresh, white, whey fondant to darken during storage. Whey solids, particularly whipped sweetened condensed whey, were found useful in the manufacture of colored fondants. An attractive center for chocolate coating was made by mixing equal parts of sweetened condensed whey and peanut butter. This center had a good peanut-

TABLE 1

Brittle "Wheyfers"

Ingredients	Working formula	Solids used
	unit weight [1]	*pct.*
Sweetened condensed whey	5,130	.....
Whey solids (39 per cent)	.....	40
Sugar (39 per cent)	.....	40
Cereal, precooked	250	5
Nuts	750	15
Total	6,130	100

[1] Grams, ounces, or pounds.

butter flavor, was not sticky, and was only mildly sweet. A hard, chewy, nut candy suitable for chocolate-coated bars resulted from cooking the ingredients of the taffy formula (Table 5) to a high temperature, about 120°C.(248°F.), adding nuts, and pouring the candy on a stone slab.

Whey solids in the form of sweetened condensed whey was substituted for egg white in marshmallow formulas requiring this ingredient. When whey completely replaced egg white, the marshmallow showed a slight weakness in body, tasted slightly salty, and turned from white to light buff in color during aging. Marshmallow made by substituting whey for about half the egg albumen which might normally be used had a good body and flavor, but it was still subject to slight changes in color during storage.

DIRECTIONS FOR MAKING WHEY CANDY

Working formulas and approximate percentage composition on a dry basis for five varieties of candy are given (Tables 1 to 5). The

total weight of the batch as given in the working formula varies between 6,000 and 7,000 grams but represents in each case 5,000 grams of solids, the weight above this figure being water carried by the different ingredients. The column headed "solids used" contains the percentage by weight on a water-free basis of ingredients which were used in the candy mixture. While these figures represent the approxi-

TABLE 2

Fudge

Ingredients	No. 1 Chocolate		No. 2 Vanilla		No. 3 Vanilla	
	Working formula	Solids used	Working formula	Solids used	Working formula	Solids used
	unit weight [1]	*pct.*	*unit weight* [1]	*pct.*	*unit weight* [1]	*pct.*
Sugar	630	12.6	.....	.....	.....	.....
Corn sirup (80 per cent)	270	4.3	360	5.75	360	5.75
Invert sirup (80 per cent)	180	2.9	180	2.9	180	2.9
Coconut fat (m.p. 110°F.)	135	2.7	135	2.7	135	2.7
Butterfat (as butter or cream)	.....	.....	135	2.7	135	2.7
Sweetened condensed whey	2,707	.....	3,510	.....	3,500	.....
Whey solids (40 per cent)	.....	21.65	.....	28.1	.....	28.0
Sugar (40 per cent)	.....	21.65	.....	28.1	.....	28.0
Water	200	.....	.....	.....	.....	.....
Cook to 121°C.(249.8°F.), draw from kettle, add:						
Corn sirup	270	4.3	360	5.75	360	5.75
Fondant (approx. 11 per cent water)	1,350	.....	1,350	.....	350	.....
Cane sirup	.....	4.8	.....	4.8	.....	1.2
Sugar	.....	19.3	.....	19.3	.....	5.0
Sweetened condensed whey	.....	.....	.....	.....	1,125	.....
Whey solids (40 per cent)	.....	.....	.....	.....	.....	9.0
Sugar (40 per cent)	.....	.....	.....	.....	.....	9.0
Chocolate	290	5.8	.....	.....	.....	.....
Powdered lactose	5	.....	5	.....	.....	.....
Vanilla to flavor						
Total	6,037	100.0	6,035	100.0	6,145	100.0

[1] Grams, ounces, or pounds.

mate composition on a dry basis of the finished candies, they do not reflect the amount of sucrose inversion or other changes incident to heating. Commercial grades of corn and invert sirups produced for confectioners and containing approximately 80 per cent solids were used.

Brittle "Wheyfers": Whip the sweetened condensed whey in a mechanical beater for about five minutes or until its volume is at least doubled. Carefully fold the cereal and nuts into the whipped sweetened whey and dry the mixture in layers about ¼ to ½ inch

thick on screening or cheesecloth so that drying takes place on both the upper and lower surfaces. Dry in a tunnel drier at about 80°C. (176°F.) for 24 hours or less, depending upon the temperature and the air circulation.

Whey Fudge: Cook with stirring, the first group of ingredients shown in the table. The temperature to use will vary between 119°C. (246°F.) and 121°C.(250°F.), depending upon the firmness of body desired. After cooking, cool the hot sirup to about 75°C.(167°F.) without stirring. Stir in the fondant, corn sirup, lactose or sweetened condensed whey, and flavoring, as given (Table 2). Continue

TABLE 3

Whipped Fudge

Ingredients	Working formula	Solids used
	unit weight [1]	*pct.*
Sugar	2,240	44.8
Corn sirup (80 per cent)	910	14.6
Invert sirup (80 per cent)	300	4.8
Water	500	.....
Cook to 133°C.(272°F.), cool to 110°C.(230°F.), add the following:		
Whipped, sweetened condensed whey	1,780	.....
Whey solids (40 per cent)	.....	14.2
Sugar (40 per cent)	.....	14.2
Vanilla to flavor		
Mix well, or whip 2 minutes, fold in:		
Chocolate, melted	375	7.4
Total	6,105	100.0

[1] Grams, ounces, or pounds.

stirring and cooling for a few minutes but not until the viscosity of the mass becomes too great for easy handling. Pour the fudge quickly into wooden forms and hold overnight in a warm room.

Chocolate fudge may be made by replacing equal parts of coconut fat or butterfat and corn sirup with chocolate in the vanilla formulas and by reversing this substitution when making vanilla fudge by formula No. 1. A strong chocolate flavor may be obtained by replacing all the coconut and butterfat in the formulas (Table 2) with chocolate.

The fondant for the fudge (Table 2) is made as follows:

Sugar	6,000 grams
Corn sirup	1,500 grams
Water	1,870 grams

Cook the mixture to 116°C.(241°F.) and cool to 40°C.(104°F.) without stirring. Crystallize the sugar during continuous stirring in a cream beater or by other mechanical means.

Whipped Fudge: Cook and cool the sugar mixture as directed in the table. Whip the sweetened condensed whey about five minutes

TABLE 4

Caramel

Ingredients	Working formula	Solids used
	unit weight [1]	*pct.*
Sweetened condensed whey	3,280	.....
Whey solids (40 per cent)	.....	26.2
Sugar (40 per cent)	.....	26.2
Condensed skim milk (30 per cent)	800	.....
Milk solids	.....	4.8
Corn sirup (80 per cent)	1,480	23.7
Invert sirup (80 per cent)	395	6.3
Coconut fat (m.p. 110°F.)	295	5.9
Chocolate	345	6.9
Vanilla to flavor		
Total	6,795	100.0

[1] Grams, ounces, or pounds.

TABLE 5

Taffy

Ingredients	Working formula	Solids used
	unit weight [1]	*pct.*
Corn sirup	2,500	40
Sweetened condensed whey	3,750	.....
Whey solids (40 per cent)	.....	30
Sugar (40 per cent)	.....	30
Water (if necessary for mixing)	150	.....
Cook with stirring to about 117.5°C. (243°F.), cool, pull, and flavor		
Total	6,400	100

[1] Grams, ounces, or pounds.

with a mechanical beater to 100 to 150 per cent overrun. Mix the cooled sirup, whipped whey, and vanilla, and carefully fold in the melted chocolate. Pour in wooden forms.

Caramel and Taffy: Cook caramel ingredients with stirring to a firm ball or approximately 119°C.(246°F.). Pour on a stone table. The taffy can be cooked to a higher temperature, then cooled and pulled.

Table 4 contains a formula for chocolate caramel. Vanilla caramel may be made by substituting various quantities of milk solids, corn sirup, and fat for the chocolate. The following two formulas give attractive vanilla caramels. No. 2 contains butterfat and a harder coconut fat than No. 1, and some of the sweetened condensed whey has been replaced by corn sirup:

	Caramel No. 1 Solids	Caramel No. 2 Solids
Sweetened condensed whey	*pct.*	*pct.*
Whey solids	26.2	22.0
Sugar	26.2	22.0
Condensed skim-milk solids	7.0	9.0
Corn sirup	23.7	28.0
Invert sirup	6.3	6.3
Coconut fat	10.6	6.7
Butterfat	.....	6.0
	100.0	100.0

DISCUSSION

Whey solids can be used in candy to partly replace skim-milk solids, sugar, and, to a lesser extent, corn sirup. The same intensity of milk flavor may be obtained in a candy when the skim-milk solids are replaced by approximately twice their weight of whey solids, while the flavor will be greatly improved if the whey is used in addition to the skim milk.

The high nutritional value of whey candy should be emphasized. Many candies can be produced which contain 20 to 30 per cent whey solids, while special formulas for candy with a whey content up to 40 per cent have been given. A 40 per cent whey-solids candy would contain approximately 1,800 Bourquin-Sherman units of riboflavin per pound in addition to the high percentages of lactose, albumin, and milk salts.

Experimental work has shown that satisfactory candy can be made from whey but the candy maker is interested also in the economic advantages of whey solids. Whey is today a by-product of little value. It seems probable that an attractive price for whey solids could be made to the candy maker, which would allow a reasonable profit for the cheese factory and the whey processor. Calculations for plain or sweetened condensed whey indicate that the cost of water removal, the chief processing expense, would be about 1.9 cents per pound of whey solids. Fresh whey may be valued at 12 cents per hundred pounds or 1.8 cents per pound of solids. This would place the value of whey solids in a condensed product suitable for candy making at 3.7 cents per pound exclusive of transportation

costs and profit to the processor. It seems reasonable to expect that the candy manufacturer would be willing to pay the price of cane sugar for whey solids or about 4½ to 5 cents per pound.

SUMMARY

An investigation of various whey products that were found suitable for the manufacture of whey candy indicated that sweetened, condensed, Cheddar-cheese or Swiss-cheese whey was, in general, the most satisfactory. When condensed without sugar, this whey was suitable for use in candy but its perishable nature was a serious disadvantage. Condensed, acid, cottage-cheese whey possessed good keeping qualities, but it required neutralization and it sometimes produced candy of inferior flavor.

It was demonstrated that excellent candy containing up to 40 per cent whey solids could be made, the whey replacing, in part, sugar, skim milk, and corn sirup. Adjustments were made in handling technique and in formulas to allow for the effect of whey upon sucrose inversion and for the development of proper body through control of the physical state of the lactose.

Directions for making various types of whey candy, including formulas which utilized the whipping properties of sweetened condensed whey, are presented.

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SOYBEAN FLOUR AS AN EMULSIFYING AGENT IN PREPARATION OF SALAD DRESSINGS

BETTY M. WATTS AND LURA MORSE
Department of Home Economics, University of California, Berkeley, California

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The efficiency of egg yolk as an emulsifying agent has long been attributed to its high phosphatid content. Since soybeans are second only to egg yolk in phosphatid content, the possibility was suggested that they also might be utilized in the preparation of salad emulsions.

Two previous reports on the use of soybean flours as emulsifying agents have come to the attention of the authors during the present research. Field, Alexander, and Sylvanus (1933) reported the preparation of a salad dressing "equal in quality to mayonnaise" from a cooked paste made of whole soybean flour and water. These emulsions were characterized by their very thick consistencies; however, pure lecithin, extracted either from soybeans or from egg yolk, had practically no emulsifying ability.

Sell, Olsen, and Kremers (1935) came to the conclusion that the effective emulsifying agent of egg yolk was not free lecithin but a loose combination of lecithin and protein which they termed "lecitho-protein." Removal of fat and uncombined lecithin from the yolk solids by benzine extraction left a much more active fraction containing the lecitho-protein. Any treatment tending to break the combination, such as alcohol extraction, destroyed the emulsifying action.

Olsen (unpublished report) then tried the same procedure on soybean flour. The fat was extracted from the flour with benzine and an artificial yolk prepared by combining the fat-free material with an equal quantity of cottonseed oil, salt, and as much water as the combined weight of flour and oil. This imitation yolk was used in the usual proportion of fresh yolk in the preparation of mayonnaise. The stiff consistency of the resulting dressing led Olsen to the tentative conclusion that the soybean paste was a much better emulsifying agent than an equal amount of egg yolk.

In both of these reports the soybean dressings seem to have been evaluated on the basis of consistencies alone. Their great thickness, as compared with egg-yolk dressings, is stressed in both cases. Preliminary experiments in this laboratory confirmed these observations; i.e., very stiff dressings were obtained with both whole or benzine-extracted soybean flour. However, under adverse conditions

of storage (widely fluctuating temperatures) oil separated from the soybean dressings more readily than from the egg yolk.

In view of these preliminary experiments as well as from theoretical considerations it seemed apparent that a quantitative evaluation of salad dressings should include not only a measure of the stiffness or viscosity, but also a comparison of their stabilities, dependent mainly upon the size of the dispersed droplets. Viscosity of the dispersions medium is not necessarily correlated with emulsifying efficiency. In fact, viscosity may be increased as much as desired by the addition of cooked-starch pastes, gelatin, etc., which by themselves have very little emulsifying ability. The added stiffness prevents or retards rising of the oil droplets and appearance of a water layer at the bottom in dressings containing a relatively low amount of oil; it is much less effective in preventing separation of an oily layer owing to an unstable emulsion of large oil droplets.

The present report is a comparison of dressings made from cooked and uncooked soybean flours, both whole and benzine-extracted, with egg-yolk and egg-white dressings made by the same method. The comparisons include camera lucida drawings of the oil droplets as well as quantitative measurements of the relative viscosity.

EXPERIMENTAL PROCEDURE

The following formula was used for all of the dressings:

Cottonseed oil	105.0 grams
Vinegar (4% acidity)	22.5 grams
Spice mix	7.5 grams
(64% sugar, 20% salt, 10% mustard, 6% paprika)	
Emulsifying agent	75.0 grams

The oil constituted 50 per cent of the total formula (a relatively light dressing).

Various concentrations of egg white, yolk, and soybean flour were used as emulsifying agents. The egg white used was a spray-dried sample previously found to be equal to fresh white when diluted to the same solids content. The dried yolk available was poorer than fresh yolk in emulsifying ability, therefore fresh yolks from eggs (U. S. specials) obtained at a local market were used.

Samples of soybean flour ground from greenish yellow beans believed to be of the Tokyo variety were used. A portion of this flour was extracted with redistilled petroleum ether until no further yellow color appeared in the solvent. One commercial solvent-extracted flour prepared from a mixture of several varieties of beans was found to be equal to the laboratory-prepared sample in emulsi-

fying ability, but a number of other commercial fat-free flours were unsuccessful, possibly because of heat treatment during the processing.

A Waring mixer (Waring Mixer Corp., New York) was used for the preparation of the dressings, since better emulsions could be obtained with this apparatus than with ordinary electric beaters. The emulsifying agent, spice mix, and vinegar were combined and placed in the mixer. Five milliliters of oil were added and the machine turned to high speed for 30 seconds. The remaining oil was run in at a uniform rate from a separatory funnel over a period of approximately three minutes with the mixer running at high speed. The ingredients were at room temperature [23 to 25°C.(73.4 to 77°F.)] when mixed. During emulsification the temperature rose to 32 to 33°C.(89.6 to 91.4°F.). The finished dressing was allowed to stand for two hours at room temperature before being tested.

Viscosity determinations were made on a Stormer viscosimeter, using a weight of 470 grams. Since the time required for 100 revolutions of the cylinder decreased with repeated measurements on the same sample, 10 successive determinations were made on each dressing. The viscosity fell rapidly during the first three to five runs, then became constant or continued to fall at a very slow rate. It is believed that the value obtained on the tenth run is a better indication than the original consistency of the probable condition of the dressing after handling and shipping. Both values are reported.

Camera lucida drawings were also made of each sample to show the relative size of the emulsified oil droplets. Magnification was the same in each case.

A series of dressings was prepared with each emulsifying agent, in which the concentration was varied from three to 7.5 grams of solid material in the 75 grams of total emulsifying agent, the difference being made up with water. Since the fresh yolk contained approximately 50 per cent of water the amount used varied from six to 15 grams. This represented a range of 1.4 to 3.6 per cent of egg or soybean solids in the finished dressing.

DISCUSSION OF RESULTS

The results of viscosity determinations on the highest concentration of each emulsifying agent used (uncooked) show the markedly higher viscosities of the soybean dressings, particularly the dressing prepared from benzine-extracted flour (Table 1). High viscosity was accompanied by greater resistance to drainage. A liquid

layer began to appear at the bottom of the egg and whole-soybean dressings within 24 hours, whereas that from the benzine-extracted flour has been stored for several months at room temperature with no detectable sign of drainage.

Camera lucida tracings of the same dressings (Fig. 1) show that, in spite of its considerably lower viscosity, the egg-yolk dressing was more efficiently emulsified than any of the others. Droplet size of

TABLE 1

Viscosity Comparisons of Egg and Soybean Dressings

Emulsifying solids in dressing		Time for 100 revolutions of Stormer viscosimeter	
		1st trial	10th trial
	pct.	*sec.*	*sec.*
Egg yolk	3.6	10.4	7.5
Egg white	3.6	14.2	12.4
Uncooked, whole, soybean flour	3.6	26.2	14.0
Uncooked, fat-free, soybean flour	3.6	167.0	29.8

the very viscous, fat-free, soybean-flour dressing was very similar to the sample prepared from an equal amount of dried egg white, while the whole soybean flour produced a slightly coarser product, undoubtedly owing to the presence of the inert oil which reduces the amount of active emulsifying agent in a given quantity of flour.

TABLE 2

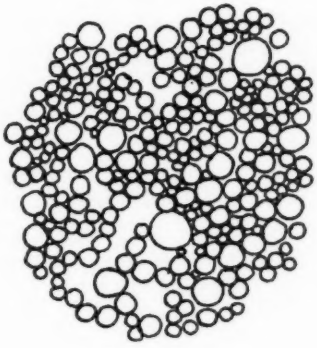
Viscosity Comparisons of Cooked and Uncooked Soybean Dressings

Soybean solids in dressings		Time for 100 revolutions of Stormer viscosimeter	
		1st trial	10th trial
	pct.	*sec.*	*sec.*
Uncooked whole flour	2.1	11.1	7.2
Cooked whole flour	2.1	92.3	20.4
Uncooked fat-free flour	2.1	19.4	10.0
Cooked fat-free flour	2.1	228.0	75.7

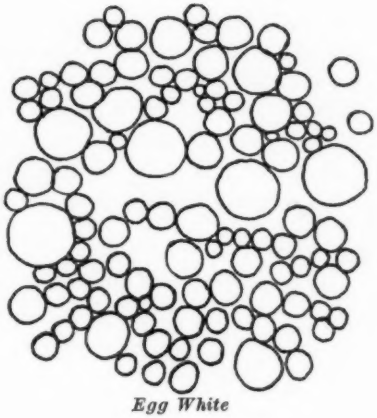
Reducing the percentage of emulsifying solids caused a decrease in viscosity and an increase in droplet size with all of the emulsifying agents; but even at the lowest level used (1.4 per cent) the egg yolk produced an emulsion equal to or slightly finer than benzine-extracted soybean flour at the 3.6 per cent level.

In comparing the cooked with the uncooked soybean dressings (Table 2) it was necessary to reduce the concentration of emulsifying solids from 3.6 to 2.1 per cent, since otherwise the cooked dressings became so thick that it was impossible to incorporate the full amount

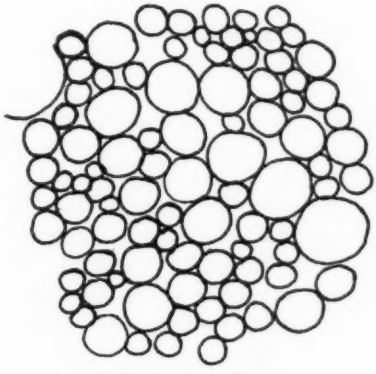
of oil. The great increase in relative viscosity on cooking is evident. The cooked dressings have been stored for several months without drainage. The consistency of the dressing prepared from the cooked benzine-extracted soybean flour was too thick to be desirable; it retained the shape of the container when turned out and showed a



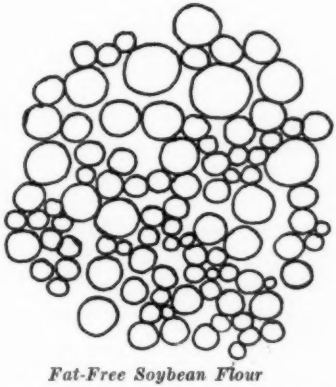
Egg Yolk



Egg White



Whole Soybean Flour



Fat-Free Soybean Flour

FIGS. 1 and 2. Camera lucida drawings of emulsions containing 3.6 per cent emulsifying solids. (Magnification 850 ×.)

slight tendency to syneresis on standing. A better product was obtained by reducing the percentage of fat-free flour to 1.4.

Cooking had no effect on the droplet size; camera lucida drawings of the cooked dressings were very similar to those of the uncooked preparations from the same quantity of flour.

From the flavor standpoint uncooked, whole-soybean-flour dressings were unacceptable. The raw-bean taste characteristic of the untreated flour was quite evident in the finished dressings. Also a strongly rancid odor developed in these dressings on standing. The uncooked, benzine-extracted, soybean flour was devoid of the raw-bean taste, but had a residual bitterness which became somewhat objectionable in the dressings containing more than three per cent of flour. Rancidity (as evidenced by detectable change in flavor) did not occur. All of the cooked dressings were excellent in flavor after several months' storage as well as when freshly made.

Egg yolk was then combined with various concentrations of benzine-extracted soybean flour with the idea of preparing a dressing containing a relatively small amount of yolk which would be finely

TABLE 3

Thinning Effect of Egg Yolk on Soybean Dressings

Emulsifying solids in dressing		Time for 100 revolutions of Stormer viscosimeter	
		1st trial	10th trial
	pct.	*sec.*	*sec.*
Egg yolk alone	1.4	5.5	3.3
Egg yolk 1.4 plus egg white	3.6	15.5	11.6
Egg yolk 1.4 plus cooked, fat-free, soybean flour	2.1	12.0	7.7
Egg yolk 1.4 plus uncooked, fat-free, soybean flour	3.6	13.8	8.9
Egg yolk 1.4 plus cooked, fat-free, soybean flour	3.6	20.7	11.9

dispersed as well as viscous. Camera lucida drawings of these combination dressings showed the droplet size to be smaller than when either emulsifying agent was used alone, as might be expected. Surprisingly, however, the viscosity of the soybean dressings, whether cooked or uncooked, decreased very greatly when combined with a small amount of egg yolk (Table 3). Concentrations of cooked soybean flour (3.6 per cent) which, when used alone, produced dressings so thick that they could not be moved by the mixer even before all of the oil had been incorporated, when combined with 1.4 per cent of egg yolk became almost as fluid as the egg yolk alone. The cause for this sharp drop in viscosity was not determined. The combination egg yolk-soybean flour dressings were very similar in viscosity and degree of dispersion to egg yolk-egg white combinations in the same proportion.

SUMMARY

Benzine-extracted, undenatured, soybean flour, heated with water, was successfully used as an emulsifying agent in the preparation of salad dressings. The emulsions obtained were less finely dispersed

than those prepared from an equal proportion of yolk solids, but were practically identical in droplet size to egg-white emulsions made over the same formula. The relative viscosity of the soybean dressings was much greater than those from either yolk or white, making possible the preparation of inexpensive salad dressings containing a low amount of oil with the soybean flour as the sole emulsifying and thickening agent.

The fat-free soybean flour was also used to replace egg white in combination with egg yolk as an emulsifying agent but the characteristic high viscosity of the cooked soybean flour was lost on addition of yolk.

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STUDIES ON THE BACTERIOLOGY OF WILTSHIRE BACON[1]
I. METHODS FOR QUANTITATIVE ANALYSIS OF CURING PICKLE

G. B. LANDERKIN
Division of Bacteriology and Dairy Research, Department of Agriculture, Ottawa, Canada

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Since 1934, studies on bacon-curing problems have been carried on by the Division of Bacteriology, Science Service, in coöperation with a local packing plant. Chief interest has been directed toward a fundamental study of the microörganisms associated with the curing of Wiltshire sides. The following report deals with the development of methods for analysis for Wiltshire pickle.

Studies on the relation of bacteria to salt have produced a voluminous literature which will not be summarized here. Much of our knowledge of the effect of salt on bacteria has been derived from investigations of bacterial life in brines, extensively reviewed by Hof (1935), and from studies of the deterioration and discoloration of fish, hides, or other products cured in salt or strong brines. The media employed have been very diverse in character and devised chiefly with the object of isolating and cultivating halophilic organisms. Thus, Hof (1935) isolated organisms from salt environments by means of saline enrichment cultures on standard laboratory media and on a bean-extract medium containing from 12 to 18 per cent sodium chloride. LeFevre and Round (1919) isolated organisms concerned in the fermentation of cucumber pickles by means of a cucumber-juice medium containing 10 per cent added salt. Schoop (1929) developed a modified fish bouillon containing 15 per cent sodium chloride for the examination of canned salt fish. Clayton and Gibbs (1927) prepared peptone, fish, and rice-water medium with 20 per cent added salt for the isolation of halophiles. Lochhead (1934) developed a milk-salt (20-25 per cent) agar medium for the isolation of halophiles from discolored hides. Numerous references to the bacteriology of salt lakes and sea water reveal many modifications of saline media. Most of the experimental work has been directed towards the isolation of specific bacterial types rather than to a study of the total flora and quantitative relationships at different salt strengths.

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Little has been published on the bacteriology of meat-curing pickles, although several reports of ham souring and related problems occur in the literature. Tanner and Evans (1933a, 1933b, 1934a, 1934b), studying the effect of meat-curing solutions on anaerobic bacteria, have shown that sodium chloride possessed the greatest activity as a preservative and that neither sodium nitrite nor sodium nitrate alone in the concentrations used commercially had much effect. Four commercial curing mixtures failed to inhibit growth or toxin formation when they were used in the proportions called for in the formulae. Sturges (1923) reported that ham-curing solutions contained a heterogeneous flora even though 15 per cent salt was present. Sturges and Heideman (1924) later classified the bacteria found in curing pickle according to their salt tolerances. Horowitz-Wlassowa (1931) studied the organisms in meat-curing brine and reported a count of 10,000 per ml. in old pickle. In most cases nutrient agar medium was used, with or without the addition of salt or curing pickle. Little information is available as to the effect of salt concentration or other factors concerned with the quantitative estimation of organisms from pickling brines. Accordingly, the present paper summarizes experience gained during five years' study of the estimation of bacteria in curing pickle.

EXPERIMENTAL PROCEDURE

Preparation of Media: Difco nutrient agar was used as the basic medium from which 5-, 10-, and 15-per cent salt agars were prepared on a weight-volume basis. All media were standardized to pH 6.7 and filtered prior to sterilization. For counts by the dilution method, Difco nutrient broth and a broth containing .1 per cent yeast-extract and .5 per cent tryptone were tested. Both contained 10 per cent sodium chloride in addition to .1 per cent potassium nitrate or .002 per cent potassium nitrite. To obtain semi-solid media from the above, .1 per cent agar was added. In addition, preliminary tests were made with pork and pickle infusion agars. The former was prepared from 500 grams of fresh pork or from a commercial desiccated product, and the latter by adding 50 per cent by volume of sterile pickle (Seitz filtered) to an equal volume of hot, sterile three-per cent agar.

Effect of Incubation Period on Development of Colonies: In preliminary tests at incubation temperatures of 37, 20, and 5°C.(98.6, 68, and 41°F.) it was found that maximum counts were obtained on all media at 20°C. The rate of colony development on media containing increasing quantities of salt varied with the salt concentration

(Table 1), where maximum colony development is taken to be 100 for each medium.

In preliminary experiments with 20- and 25-per cent salt agars the lag in colony development was so great that under normal conditions of incubation salt crystals formed and made counting very difficult. This, and the fact that bacteria counts were in all cases lower on the 20- or 25-per cent salt agar than on 10- or 15-per cent salt, precluded further analyses with these media. Although maximum counts were not obtained on nutrient agar plates until the third day at 37°C. or the sixth day at 20°C., it was decided to standardize on incubation periods of two and four days, respectively, to facilitate plant control. With five-per cent salt agar two weeks' incubation was selected, and three weeks allowed for 10- and 15-per cent salt agar.

TABLE 1

Effect of Length of Incubation on Relative Counts at 20°C.(68°F.)
(Average of 11 samples)

Medium	Time in days				
	2	4	8	14	21
Nutrient agar	[1]	87	100	100	100
5% Salt agar	[1]	17	72	94	100
10% Salt agar	[1]	[1]	[1]	87	100
15% Salt agar	[1]	[1]	[1]	87	100

[1] Colonies undeveloped or ill defined.

Effect of Salt Concentration on Plate Counts From Pickle: Only well-mixed representative samples of Wiltshire pickle taken before and after filtering were selected for quantitative analytical studies. At this stage the composition of different samples was essentially the same, the used pickle being rebuilt and mixed prior to filtering. The effect of salt concentration on plate counts from pickle is illustrated (Fig. 1), where the maximum logarithmic-average bacteria count (on 10-per cent salt agar) is taken to be 100. Increasing or decreasing the amount of salt in the medium reduced the number of bacteria obtained. On 15-per cent salt agar only 49 per cent of the maximum count was obtained and on five-per cent salt and nutrient agar the percentages developing were 59 and 8.8, respectively. On the latter medium at 37°C. only 1.6 per cent of the number of colonies developed.

While the bacteria counts on nutrient and salt agars showed a definite relationship, not only on the 50 selected samples but on more than 200 samples taken over a period of five years, it was observed that with improvements in plant sanitation the spread between nutri-

ent and 10-per cent salt agar counts became greater. This is believed due to lessened contamination (by non-halophilic organisms) of the sides going into pickle, since the trend was a reduction in nutrient agar counts rather than an increase in counts on 10-per cent salt

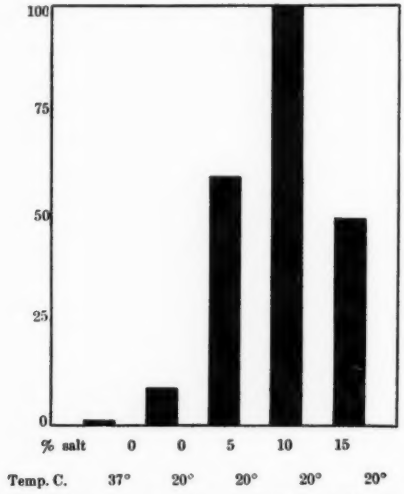

FIG. 1. Ratios of bacteria counts from Wiltshire pickle on nutrient and various salt agars. (Average from 50 samples, saline dilutions with salt agar.)

agar. The increasing difference is shown (Table 2), where the ratios of logarithmic-average bacteria counts from samples taken in the late autumn of four consecutive years are given.

TABLE 2

Ratios of Bacteria Counts From Wiltshire Pickle in Four Consecutive Years

Year	Month	Number of samples	Nutrient agar 37°C.	Nutrient agar 20°C.	10% Salt agar 20°C.
1935	Nov.-Dec.	8	1.5	7.7	100
1936	Oct. -Nov.	8	1.3	7.9	100
1937	Oct. -Nov.	7	0.8	4.4	100
1938	Oct.	11	0.5	3.4	100

Bacteria Counts on Miscellaneous Media: Irregular results were obtained with liquid and semi-solid media inoculated in serial dilutions in duplicate. Contrary to general experience with dilution methods the probable numbers of bacteria (calculated from McCrady's tables) were lower in all cases than plate counts on 10-per

cent salt agar. This abnormality may be subject to qualitative explanation, for on 10-per cent salt agar the flora consists largely of obligate halophilic microörganisms which grow very indifferently if at all in liquid media.

In comparison with salt agar, greater counts were obtained on laboratory prepared pork infusion and lesser counts on the desiccated product (Table 3).

TABLE 3

Ratios of Bacteria Counts From 16 Samples of Wiltshire Pickle

Plating medium	Ratio
10% Salt agar	100
10% Salt agar + .1% KNO_3	126
10% Salt, pork agar (lab.)	150
10% Salt, pork agar + .1% KNO_3	155
10% Salt, pork agar (des.)	65
10% Salt, pork agar + .1% KNO_3	118

The addition of potassium nitrate to these and other media introduced marked variations in bacteria counts. Generally, but not always, significant increases in colony development resulted. Potassium nitrite was inhibitory, 90 per cent of those colonies capable of developing on 10-per cent salt agar being inhibited by concentrations greater than .4 per cent in the medium. Bacteria counts on 50-per cent pickle-infusion agar were in all cases lower than on 10-per cent salt agar.

Effect of Salinity of Dilution on Bacteria Counts: For quantitative analysis "square" Pyrex bottles containing 90 ml. of diluent were employed. In preliminary analyses no significant differences in bacteria counts were obtained when tap water or physiological saline dilutions were used. When higher salt concentrations were tested, however, it was apparent that the salinity of the dilution had a marked effect upon the counts obtained on high salt media. The change in osmotic pressure involved in the transfer from pickle containing approximately 30 per cent (wt./vol.) of sodium chloride to physiological saline renders incapable of developing many cells otherwise viable on salt agar. This effect is illustrated (Fig. 2 and Table 4).

TABLE 4

Average Bacteria Counts From Seven Samples of Wiltshire Pickle With Tap Water and 10-Per Cent Saline Dilutions

Dilution system	Medium	
	Nutrient agar	10% Salt agar
Tap water	41,500	27,600
10% Saline	42,300	941,400

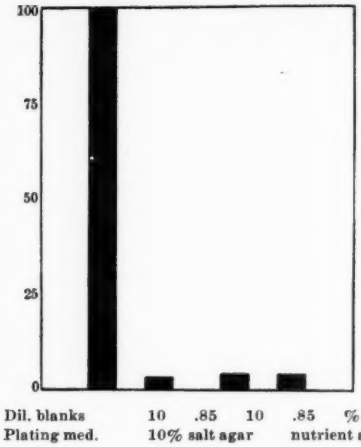

FIG. 2. Ratios of bacteria counts from Wiltshire pickle on nutrient and 10-per cent salt agar with physiological and 10-per cent saline dilutions. (Average from seven samples.)

The lethal effect of tap water or physiological saline dilutions upon certain bacteria was repeatedly observed in analyses of Wiltshire pickle and appeared to be instantaneous and little affected by the time the cells were exposed to the diluent as shown (Table 5).

TABLE 5

Effect of Duration of Exposure to Tap-Water Dilutions on Salt-Agar Counts From Pickle

Plating medium	Dilution	Time	Bacteria count
		min.	
10% Salt agar	10% Saline	15	2,200,000
	Tap water	15	50,000
	Tap water	1¾	50,200
	Tap water	1¼	59,200
	Tap water	¾	55,200

Likewise the effect was observed whether tap water was used for all dilutions or only in part (Table 6).

The direct relation between salinity of dilutions and bacteria count on 10-per cent salt agar is shown (Fig. 3), where the ratios of logarithmic-average bacteria counts from four samples of Wiltshire pickle are plotted against the salt concentration of dilutions. It will be noted that as the salinity decreased from 10 per cent the lethal

effect became greater and was greatest from 0 to four per cent. No increases were noted when sterile pickle or concentrations greater than 10-per cent saline were used for diluting samples. While most of the experimental work centered on pickle bacteria developing on 10-per cent salt agar, the effect was observed on five-per cent salt agar and

TABLE 6

Effect of Using 10 Per Cent Saline Dilutions in Part on Salt-Agar Counts From Pickle

Medium	Dilution system	Bacteria count	
		Test 1	Test 2
10% Salt agar	10% Saline all dilutions	6,000,000	252,000
	10% Saline first dilution only	320,000	11,500
	Tap water all dilutions	405,000	13,000

all higher concentrations, and in platings from slime on mature Wiltshire sides as well as from the curing pickle. It would appear that those organisms inhibited by non-saline dilutions are halophilic in

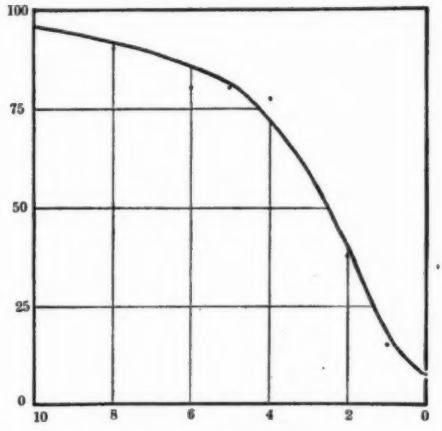

FIG. 3. Ratios of bacteria counts from Wiltshire pickle on 10-per cent salt agar according to salinity of dilution. (Average from four samples.)

nature rather than salt tolerant, for the percentage of halophilic organisms in pickle capable of developing on nutrient agar is in line with the relation of the reduced count on 10-per cent salt agar and the normal count on nutrient agar (Fig. 4).

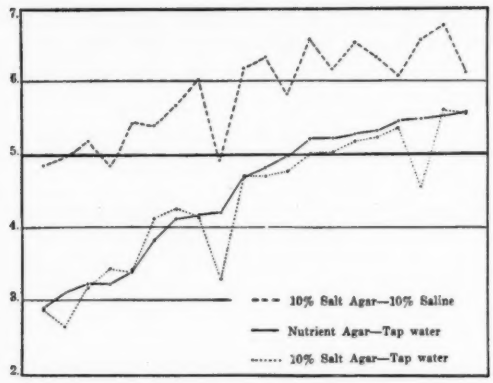

FIG. 4. Logarithmic-average bacteria counts from 20 samples of Wiltshire pickle on nutrient and 10- per cent salt agars with saline and tap-water dilutions.

DISCUSSION

The function of salt lies in its selective and inhibitive action towards certain types of bacteria. This action is most apparent in the study of bacteria developing on various salt media. The correlation between bacteria counts on nutrient and salt agars indicates the stability of an established pickle flora and may serve as an index of plant sanitation. It is generally believed that the role of organisms in the pickle is directly concerned with the reduction of nitrates and the little-understood alterations which differentiate a cured product from one merely salted. From the results obtained in this laboratory there is little evidence to show that bacteria are active in the pickle solution, for the maximum counts were obtained on media containing less than half the concentration of salt present in the pickle, and in liquid media of high-salt concentrations growth is negligible. It would appear that bacterial activity is confined largely to the immediate proximity of the side and even within the membranes of the side itself, for it is believed that here in the zone of salt transfer concentrations most suitable for the development of the pickle flora are to be found.

From the results obtained in varying the salinity of dilutions it would appear that there are extreme variations in the vitality of organisms in pickle, since over 90 per cent of the bacteria capable of developing on 10-per cent salt agar are destroyed on transfer to non-saline dilutions. According to Garrard and Lochhead (1939) certain substances are present in pickle which lessen the toxic effect of high-salt concentrations. Thus it is possible for certain non-salt-

tolerant organisms contaminating meat prior to curing to survive the pickling process and contribute to later storage defects. Pickle then may be regarded as containing not only obligate halophilic and salt-tolerant organisms, but non-salt-tolerant forms which may become active only after removal of sides from the high-salt environment of pickle. Thus the value of pickle analysis lies not only in the correlation of bacterial density with changes in the pickle but also with bacterial changes subsequent to curing as well as with pre-curing contamination. The existence of groups of diverse salt relationships in pickle necessitates the use of more than one medium for quantitative bacteriological analysis. A high bacteria count on nutrient agar or media containing less than five per cent of salt is apparently undesirable in pickle or at any stage in the cure, but there appears to be no great objection to moderate counts on 10-per cent salt agar.

In plant practice the bacterial content of curing pickle is limited by sanitary precautions and reduced by heating, skimming, settling, and filtering. The three great factors controlling bacteria in the pickle are temperature, salinity of pickle, and duration of the cure.

CONCLUSIONS

Experimental studies on Wiltshire pickle have shown that the rate of colony development and the total number of colonies varied with the salt concentration of the medium. Incubation of plates at 20°C.(68°F.) resulted in greater counts than at 37 or 5°C.(98.6 or 41°F.), and at 20°C. maximum bacteria counts were obtained on 10-per cent salt agar. Bacteria counts on nutrient, 5-, 10-, and 15-per cent salt agars showed a definite relation to each other on over 200 samples taken over five years. The ratio between nutrient and 10-per cent salt agar counts widened slightly, however, with increased plant sanitation.

Modifications of saline media including liquid and semi-solid dilution counts revealed fewer organisms than salt agar, and the addition of nitrite proved inhibitory. When nitrate was added to salt agar, irregular and unpredictable increases in plate counts were observed. Pickle infusion agar proved to be a poor medium.

To obtain maximum counts on salt media, saline dilutions were found to be essential, for over 90 per cent inhibition on 10-per cent salt agar resulted when physiological or tap-water dilutions were employed. The reduced counts on the salt agar were of the same order as counts obtained on nutrient agar and point to the elimination of a specific group of halophilic bacteria.

The five years' study of methods of analysis for Wiltshire pickle has shown that the flora is heterogeneous and requires at least two

distinct media for its enumeration, i.e., nutrient agar to indicate the degree of contamination and 10-per cent salt agar to reveal the natural or halophilic flora.

ACKNOWLEDGMENT

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"OXIDASE REACTION" OF BACTERIA IN RELATION TO DAIRY PRODUCTS

C. H. CASTELL AND E. H. GARRARD
Department of Bacteriology, Ontario Agricultural College, Guelph, Ontario

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Relatively little attention has been paid to the "oxidizing capacity" of microörganisms in relation to the spoilage of dairy and other food products. Many bacteria have this ability to a marked degree. Recognition of this characteristic along with proteolytic, lipolytic, acid-forming, and other familiar bacterial activities would give a more accurate picture of bacterial action in the spoilage of food.

In their work on the action of microörganisms on fats, Jensen and Grettie (1937) have shown that many of the so-called lipolytic organisms produce both lipases and oxidases. Several years' practical experience in this laboratory have indicated that most of the lipolytic organisms considered to be the cause of various types of rancidity show a marked oxidase reaction. Other organisms, in which enzymes capable of hydrolyzing fats are negligible or absent but which cause taints in butter, have been found to be strongly oxidase-positive. With these ideas in mind, a general survey has been made of the oxidase activity of some 30 species of organisms which are commonly or occasionally found in dairy products. These results are compared with the action of the same organisms on fats and their relative destructiveness in dairy products.

THE OXIDASE TEST

The procedure followed for detecting oxidase-producing bacteria is based upon the methods described by Gordon and McLeod (1928) and Ellingworth, McLeod, and Gordon (1929). This consists of flooding petri plates in which organisms are growing with a .5 per cent aqueous solution of either p-aminodimethylaniline hydrochloride or tetramethyl-p-phenylenediamine hydrochloride. As soon as the agar surface has been completely covered with the dye solution it is poured off, and the petri plates are left with their covers removed. Freshly prepared, these dye solutions are almost colorless. After contact with these dyes, oxidase-forming colonies very shortly assume a deep color. With the tetramethyl compound this color is purple, and with the dimethyl compound it is rose-red.

There are several differences between these two indicators. The tetramethyl compound changes to a colored form at a lower Eh and therefore is more sensitive and acts more quickly. With it, many strongly oxidizing colonies take on a color almost immediately. Others, especially those submerged in the medium, react more slowly. In this laboratory, with this dye, final observations are made at not more than ten minutes after the plates have been flooded. With some organisms further action of the same colony which produced the color may reduce it to a colorless form again. In other words, the reaction of the tetramethyl dye, although more sensitive, is not always permanent.

The dimethyl compound is much less costly, and for that reason has been more frequently used in routine work. It is slightly less sensitive and therefore reacts more slowly. There are a few species of weakly oxidizing organisms which are positive to the tetramethyl compound and negative to the dimethyl compound. These are rare. This dye (the dimethyl) also differs from the former in being permanent in its reaction. Instead of decolorizing, further oxidation changes the rose-red color to a deep permanent black.

Exposure to the atmosphere alone will sufficiently oxidize these dyes so as to produce their colored forms. The medium on which the dyes are flooded will also become colored, but much less quickly than the color changes produced by the oxidase-positive colonies. Some colonies not only do not oxidize the dye but also set up an "area of reduction" in the surrounding medium, which remains colorless long after the dye has been oxidized in the other portions of the agar.

EXPERIMENTAL PROCEDURE

Reaction of Pure Cultures: Thirty pure cultures were streaked in duplicate on sterile beef extract agar in petri plates. After 24 hours' incubation at 25°C.(77°F.) they were flooded in the usual manner with .5 per cent of the tetramethyl and dimethyl dyes. Similar tests were made after three and seven days of incubation. The oxidase tests divide these organisms into distinct groups: (a) those which are strongly positive, (b) those which are slightly positive, (c) those which are doubtful, and (d) those which are definitely negative (Table 1). By far the most difficult to observe are those which fall in the third group. This includes *Escherichia coli* and some of the spore-forming soil organisms. Not only are they difficult to judge, because of the weakness of the color reaction and the slowness with which it appears, but they also tend to be variable, sometimes reacting positively and at other times negatively. The reactions of the organisms in the other groups are constant. For the purpose of com-

parison, other characteristics of these cultures are also listed beside the oxidase reactions (Table 1).

For lipolysis, a butterfat emulsion agar was used and those colonies which gave a blue color to fat globules when the plates were

TABLE 1

Oxidase, Gram, Proteolytic, Lipolytic, and Other Reactions of 30 Pure Cultures of Bacteria

Organism	Oxidase reaction [1]		Proteolysis	Lipolysis	Acid from lactose	Gram
	Tetramethyl	Dimethyl				
Achromobacter lipolyticum	+++	++	+	+	—	—
Achromobacter putrefaciens	+++	++	+	—	—	—
Pseudomonas fluorescens	+++	++	+	+	—	—
Pseudomonas fragi	+++	++	+	—	—	—
Pseudomonas mucidolens	+++	++	+?	+	+	—
Pseudomonas aeruginosa	+++	++	+	+	—	—
Alcaligenes faecalis	+++	+	+	—	—	—
Alcaligenes lipolyticus	++[2]	?	+	—	—	—
Brucella abortus	++	+	—	—	—	—
Escherichia coli	+	+	—	—?	+	—
Escherichia communior	—	—	—	—	+	—
Aerobacter cloaceae	—	—	—	—	+	—
Aerobacter aerogenes	—	—	—	—	+	—
Proteus vulgaris	—	—	+	—	—	—
Salmonella paratyphi	—	—	?	—	—	—
Bacillus subtilis	+[2]	—	+	—	—	+
Bacillus graveolens	+[2]	—	+	—	—	+
Bacillus panis	—	—	+	—	—	+
Bacillus mesentericus	+[2]	—	+	—	—	+
Bacillus cereus	+[2]	+	+	—	—	+
Clostridium butyricum	—	—	+	—	—	+
Streptococcus liquefaciens	—	—	+	—	+	+
Streptococcus lactis	—	—	—	—	+	+
Streptococcus mitior	—	—	+	—	+	+
Staphylococcus aureus	—	—	—	+	+	+
Staphylococcus citreus	—	—	+	?	+	+
Micrococcus conglomeratus	—	—	—	—	+	+
Sarcina lutea	—	—	+	—	—	+
Diplococcus capsulatus	—	—	—	—	+	+
Lactobacillus bulgaricus	—	—	—	—	+	+

[1] Tests made with streak cultures on beef extract agar after 24 hours' incubation.
[2] Color disappears after a very short period.

flooded with Nile blue sulphate, were considered positive. Proteolysis was detected by a clearing in the medium adjacent to streaks or colonies grown on beef extract agar to which five per cent of milk has been added.

Age of Cultures: The length of the incubation period previous to flooding the plates with the dyes has a definite effect on the action

of the test. Older colonies show a much sharper reaction. Results of the oxidase tests on 20 pure cultures after 24, 48, and 96 hours of incubation are given (Table 2). These were streaked on beef extract agar and tested with the tetramethyl dye. The observations were made within two or three minutes after flooding the plates. In several cases, before ten minutes had elapsed oxidase-positive colonies had completely lost their color, and observations made at two and

TABLE 2

Oxidase Reaction of 20 Pure Cultures After 24, 48, and 96 Hours of Incubation, Using Tetramethyl Dye

Organism	24 hours	48 hours	96 hours
Pseudomonas aeruginosa	+++[1]	+++	+++
Alcaligenes faecalis	+++	+++	+++
Alcaligenes lipolyticus	+[2]	++[2]	+++[2]
Bacillus cereus	+[2]	+++	++
Bacillus mesentericus	+[2]	++[2]	+[2]
Bacillus subtilis	+[2]	++[2]	+[2]
Escherichia coli	+?	+	+
Aerobacter cloaceae	—	+	?
Aerobacter aerogenes	—	+	—
Proteus vulgaris	—	+[2]	+
Streptococcus liquefaciens	—	—	—
Clostridium butyricum	—	—	—
Diplococcus capsulatus	—	—	—
Escherichia communior	—	?	—
Bacillus panis	—	—	+[2]
Salmonella paratyphi	—	—	—
Sarcina lutea	—	—	—[4]
Streptococcus mitior	—	—	—
Staphylococcus aureus	—	—	—
Staphylococcus citreus	—	—	—[3]

[1] +++ Very strong reaction; ++ strong reaction; + weak reaction; — negative reaction. [2] Color of colony quickly fades. [3] Narrow band of color surrounds colony for several minutes. [4] Small + area in center of colony.

24 hours after adding the dye showed that many others, formerly negative, had assumed a color.

A similar phenomenon is demonstrated in the reaction of various portions of the colony. Very few colonies remain completely colored. (*Achromobacter putrefaciens* and certain strains of *Pseudomonas fluorescens* are the exceptions). Even the most oxidase-positive colonies, after a very short period, appear to have a colorless margin. The central portion of the colony is always stained the deepest, and the areas of most recent growth are stained more lightly. When dilute hydrogen peroxide is poured over these colonies it shows that the most rapid reduction, as indicated by the formation of bubbles,

takes place in the unstained border regions of the colonies. Similarly, when conditions are arranged so as to prevent the dyes from inhibiting bacterial action, fresh growth appearing on the plates after the application of the dyes is at first oxidase-negative.

It will be noted that these variations in the activity of the dye in relation to the length of the incubation, do not interfere with making observations on those which are strongly oxidase-positive or those which are negative. It affects mainly species which are either weakly positive or variable organisms of spore-forming soil types, and members of the coli-aerogenes and related groups.

Reaction of Other Types of Microörganisms: Five cultures of Actinomycetes, isolated from various dairy products, all reacted negatively to both dyes. The majority of molds tested were also negative; however, a culture of Oidium, Penicillium, and an unidentified white mold, reacted quite positively. It is interesting to note that the spores of this particular Penicillium were also oxidase-positive. Fifteen species of yeast, including *Willia anomala, Torula sphaerica,* a common pink Torula, a Pichia, a Schizosaccharomyces, *Saccharomyces cerevisiae* and *Saccharomyces ellipsoideus,* and other unidentified white yeasts isolated from butter and cheese, all reacted negatively to the oxidase test.

Practical Application of Oxidase Test: In much of the routine and investigational dairy bacteriology carried on in this department during the last few years, the oxidase test has been used as a regular diagnostic procedure. Some of the results are not only of interest, but also suggest the value of the more frequent use of this test in many phases of dairy bacteriology. Two or three examples will illustrate this:

1. In examining the method described by Williams (1939) for preserving cream by the addition of salt, attempts were made to determine the changes it produced in the microflora of the cream. These results have been partially described elsewhere by Castell and Garrard (1939). One of the most significant of these observations is the fact that when sufficient salt is added to preserve the cream it almost completely inhibits the growth of the oxidase-positive bacteria. The addition of salt apparently has less effect in reducing the total counts in the cream than it did in shifting the proportion of oxidase-positive and oxidase-negative organisms.

2. Plate counts made from cheddar cheese during various stages of its curing, show a microflora similar to the salt-preserved cream, in that the oxidase-positive organisms were either insignificantly few or entirely absent. Experimental cheese was made from milk heavily inoculated with organisms of the oxidase-positive group *(Ps. fluores-*

cens, Ps. fragi, Ach. lipolyticum). Samples were cured for six months at 4.5°C.(40°F.), 10°C.(50°F.), and 15.5°C.(60°F.) and then graded by the Federal cheese graders. Four of these cheese samples were graded the same as the control; four lost only one point, and one lost two points. Bacteriological examination of the cheese at the time of grading failed to show the presence of a single colony of the original contaminants. Further investigations along this line indicate that the oxidase-positive, gram-negative group of organisms, which cause various types of rancidity in butter, have little or no effect on cheddar cheese; and that most off-flavors of microbial origin in cheese are caused by members of the oxidase-negative group, including yeasts, anaerobes, and Cocci.

3. A list of the organisms producing the more serious defects in the flavor of butter (and similar fatty foods) is composed almost wholly of the strongly oxidase-positive bacteria. With this in mind, during the past two years analyses of all samples of water, cream, butter, buttermilk, churn washings, etc., have included the oxidase test. To date, not a single case of surface taint, cheesy flavor, limburger flavor or rancidity has been found where the oxidase-positive bacteria were not abnormally numerous. In several instances where it has been shown that water has been the source of butter trouble, oxidase-positive organisms have been abnormally numerous.

DISCUSSION

In the past the oxidase test has been used mainly for the differentiation of certain pathogenic bacteria. As it is based on the oxidizing capacity of the organisms, an important activity not included in other common tests, it appears reasonable that it should have a wider application, especially in the study of microbial spoilage of foods.

The test is easily made and has a great advantage in that it can be employed on plates prepared for ordinary counts or other purposes.

There is no doubt that the more spectacular and more easily controlled oxidation of dairy products and other fatty foods by non-living catalysts has eclipsed the more complex action of microbial oxidases, which are rarely found except in the company of other types of enzymes. As Jensen and Grettie (1937) have suggested, food chemists would do well to give greater consideration to microbial action in studying oxidative as well as hydrolytic rancidity. This test is a very convenient method for identifying bacteria bringing about the oxidation changes.

From the observations described in this paper, organisms of the Pseudomonas and Achromobacter genera are the most strongly oxi-

dizing types; those belonging to Alcaligenes and Brucella are somewhat less, although still strongly positive; members of the Aerobacter, Escherichia, and Proteus, are weakly positive, variable, or negative; the Bacilli also vary from weakly positive to negative; while the Cocci and the only anaerobe represented are definitely oxidase-negative.

It is interesting to note that all organisms which are *strong oxidizers* are gram-negative and those which are definitely *not strong oxidizers* are gram-positive. Further emphasis is laid on this point by the observations of Gordon and McLeod (1928), Thompson (1936) and others, that *gram-negative Cocci* of the Neisseria and Hemophilus genera are strongly oxidase positive. Anaerobes examined by Gordon and McLeod were all oxidase negative. This bears out the evidence brought forward by Stearn and Stearn (1930) that in general, gram-negative organisms react differently towards the oxidation-reduction potential of their environment from those which are gram-positive.

That the Pseudomonas and Achromobacter are strong oxidizers should be of interest to those working with butter, lard, chilled beef, and other fatty products, both because of the numerous types of rancidity and off-flavors produced by these organisms and because they frequently are able to grow actively at storage and refrigeration temperatures. Lea (1938) maintains that organisms of these two genera, together with molds and yeasts, are largely responsible for spoilage in chilled meat. The activity of these same gram-negative organisms in the spoilage of milk, butter, and some other dairy products is too well known to need mention.

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EFFECT OF HEATING ON THE CLEARING OF CITRUS JUICES

M. A. JOSLYN AND A. SEDKY
Division of Fruit Products, University of California, Berkeley, California

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Among the colloids present in fruit juices the pectic substances predominate. These substances may be converted into an insoluble gel through the action of pectin methoxylase in the presence of calcium or other alkaline earth salts and so removed, according to Kertesz (1936). Insoluble flocculent precipitates form also when pectins are hydrolyzed by enzymes present in the "pectinase" complex, Willaman and Kertesz (1931) and Kertesz (1930). The mechanism of the transformations occurring in the latter case is still imperfectly understood. The insoluble residue formed closely resembles lignic acid, obtained by Ehrlich and Schubert (1926) by acid decomposition of pectin. The available data which has been summarized by Kertesz on the heat inactivation of pectic enzymes indicates marked difference among the complexes studied. Thus protopectinase is inactivated by heating for 20 minutes at 48 to 51°C.(118.4 to 123.8°F.); pectinase in 20 minutes at 60°C.(140°F.) or 30 minutes at 55°C.(131°F.); pectase in 20 minutes at 68 to 70°C.(154.4 to 158°F.), although Kertesz (1936) found that for tobacco pectase, heat inactivation occurred in two minutes at 90°C. (194°F.) or 1.5 minutes at 100°C.(212°F.). The pH optima reported in the literature are five to six for protopectinase, about three for pectinase, and about six for pectase activity.

Kertesz (1939) has reported recently that the pectin-methoxylase of tomatoes is completely inactivated by heating the juice to 80°C.(176°F.) for 45 seconds. The sensitivity of the enzyme towards heat increased with decrease in pH. Thus inactivation was reported on heating for two minutes at about 70°C. for pH 4.4, at 55°C. for pH 2.5, and at 50°C.(122°F.) for pH 1.1. At pH 6 no inactivation was found for a heating period of two minutes at the highest temperature used, 70°C.

There have been reported but very few studies on the activity of pectic enzymes in citrus juices, the nature of the changes brought about by them in the natural citrus pectins, and the relative rates of heat inactivation of the systems involved. Cruess (1914) observed that fresh orange juice will not filter easily and is difficult to make

bright by filtration until it has stood for 24 to 72 hours. The fresh juice filtered slowly and the filtrate was cloudy, whereas after storage for the proper period of time it filtered easily and gave a clear filtrate. A sample of juice was observed to become jelly-like in a few hours after expression, but in two days the gel coagulated and settled out, carrying down all suspended material and leaving the supernatant liquid clear. Cruess (1914) postulated the presence of a clearing and clotting enzyme and pointed out that pasteurized juice will not clear but will remain cloudy. Heating the juice in the bottle to 85°C.(185°F.) prevented it from clearing during storage at room temperature for seven months. Wilson (1925) reported that lemon waste after grinding should be heated to 95 to 98°C. (203 to 208.4°F.) for about 10 minutes "to destroy pectinase, which converts pectin to pectic acid."[1] Norris (1926) reported the formation of a smooth jelly in orange juice which did not appear when the juice was heated for one or two hours at 90 to 100°C.(194 to 212°F.).

In connection with their investigations on the preservations of citrus juices, Joslyn and Marsh (1933) reported that in these juices there was a rapid settling of the chromatophores and coarse pieces of carpellary membrane leaving a cloudy supernatant liquid. In course of storage there was observed a clearing of the supernatant liquid, the deposition of a white fluffy material on the pulp particles, and a clotting or a curdling of the latter. The results of preliminary investigations made by them of the heat inactivation of the enzymes responsible for these changes, have been reported in part by Joslyn and Marsh (1933, 1934).

Observations of the samples prepared in 1933 and 1934 were continued (by Marsh) and final results are reported here. In addition results were obtained on grapefruit and lemon juices.

EXPERIMENTAL PROCEDURE

In the original experiments of Joslyn and Marsh (1933, 1934) Valencia oranges shipped from Riverside in August, 1933, and Washington navel oranges shipped from Riverside in March, 1934, were used. The juice was extracted by burring, strained, deaerated, and then centrifuged to remove chromatophores and other tissue particles in coarse suspension. Sodium benzoate was added to the centrifuged juice of uniformly cloudy appearance to give a final concentration of .3 per cent. This juice was dispensed in 10-c.c. portions onto thin-walled glass tubes 5 x ⅝ inches in size, which were heated in a water

[1]Apparently he was referring either to pectin methoxylase or pectase, or may have not meant pectic acid.

bath for 0, 2, 4, 6, 8, and 10 minutes at 65, 70, 75, 80, 82.5, and 85°C. (149, 158, 167, 176, 180.1, and 185°F.), respectively; and for 0, 1, 2, 3, 4, and 6 minutes at 87.5, 90, 95, and 100°C. (189.1, 194, 203, and 212°F.), respectively. The 0 time was taken as the time for the temperature of the juice in the test tube to reach 1°C. below bath temperature; this was approximately three minutes. Immediately after removal from the water bath the heated juice was promptly cooled by immersion of the tubes in ice water. The tubes were then stoppered with corks sterilized with mercuric chloride and stored at room temperature. Three tubes were removed at each time interval.

TABLE 1

Heat Inactivation of Clearing Enzymes in Valencia Orange Juice

Temperature	Heating required to prevent clearing at various temperatures for storage					
	Days stored					
	11	22	36	72	241	513
°C.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
65	6	>10	.....	.....	.....	.....
70	4	>10	.....	.....	.....	.....
75	0[1]	>10	.....	.....	.....	.....
80	0	>8 <10	>10	.....	.....	.....
82.5	0	>4 <6	>6 <8	>8 <10	>10	.....
85	0	>0 <2	>4 <6	>6 <8	>6 <8	>8 <10
87.5	0	0	0	0	>0 <1	>1 >2
90	0	0	0	0	0	0
95	0	0	0	0	0	0
100	0	0	0	0	0	0

[1] 0 indicates that the exposure required to bring the temperature of the juice in the test tube to bath temperature (about three minutes) was sufficient to inactivate clearing enzymes.

In the subsequent tests oranges, grapefruit, and lemons were secured on the Berkeley market in August, 1937; each fruit was peeled and pulp and peel were ground separately and mixed together. The juice was then pressed out through a clean cheesecloth and centrifuged for 10 minutes. The clear portion was separated from the sediment and to it .3 per cent of sodium benzoate was added. The pH of the juice from each type of fruit was determined; that of oranges was 4.2; that of grapefruit, 4.0; that of lemons, 2.2.

The juice from each fruit was divided into two lots. With oranges and grapefruit, one lot was left at its natural pH value and the pH of the other was adjusted to that of lemons by using concentrated citric acid. In lemons, one lot was left at its natural pH; and by the addition of sodium hydroxide, the pH of the other was adjusted to a pH value of 4, which is approximately the pH range of orange

and grapefruit juices. Thus, a comparison of the effect of varying the pH value for each fruit between the two ranges was possible. Five cubic centimeters of either lot for each fruit were placed in several thin-walled test tubes of equal diameter (10 x 1.4 cm.). Test tubes containing the juice at either pH for each fruit were heated in a water bath for intervals increasing by one minute from one to 10 minutes at temperatures progressively increased by 5° from 60 to 100°C.(140 to 212°F.). The time required for any test tube to arrive at a given temperature was one minute. These test tubes were tightly corked, labelled, and kept at room temperature.

DATA AND DISCUSSION

The samples of the first lot were observed periodically for a period of over a year and the results summarized (Tables 1 and 2).

TABLE 2

Heat Inactivation of Clearing Enzymes in Navel Orange Juice

Temperature	Heating required to prevent clearing at various temperatures for storage			
	Days stored			
	5	85	128	351
°C.	*min.*	*min.*	*min.*	*min.*
80	0[1]	>5 <10	>5 <10	>20 <40
82.5	0	>5 <10	>5 <10	>5 <10
85	0	0	0	>2 <4
87.5	0	0	0	>0 <1
90	0	0	0	0
95	0	0	0	0
100	0	0	0	0

[1] 0 indicates that the exposure required to bring the temperature of the juice in the test tube to bath temperature was sufficient to inactivate clearing enzymes.

The data indicate the progressive clearing of the juices during storage so that to prevent clearing, the temperature to which the juice is to be heated must be higher and the time it is to be held at that temperature longer, the longer the time of storage. Thus to remain cloudy for 513 days Valencia orange juce must be heated for over 10 minutes at 82.5°C., between eight and 10 minutes at 85°C., two minutes at 87.5°C., and just brought to 90°C. To remain cloudy for 351 days, navel orange juice must be heated over 20 minutes at 80°C., 10 minutes at 82.5°C., four minutes at 85°C., one minute at 87.5°C., and just brought to 90°C. The clearing enzymes in navel juice apparently are less heat resistant than in Valencia juice, thus it is necessary to heat the juice at 85°C. for four minutes for the former and for over eight minutes for the latter to prevent clearing.

In the second series of tests it was observed that in all the samples before storage there was a slight turbidity in the orange juice, more turbidity in the grapefruit juice, and much more in the lemon juice. Further observations after three weeks showed in all cases precipitation of some of the suspended matter in the juice, accompanied by slight clarification. In comparing samples from any one fruit at different pH values or at different temperatures, there was no observable change in the degree of clarification. Observations

TABLE 3

Effect of Heating at pH 2.5 and 4.2 on Clearing of Orange Juice

Temperature of bath	pH	Heating to prevent clearing at various temperatures	
		After 6 months	After 12 months
°C.		min.	min.
60	2.5	>10	>10
	4.2	>10	>10
65	2.5	>10	>10
	4.2	>10	>10
70	2.5	>10	>10
	4.2	>10	>10
75	2.5	8	>10
	4.2	>10	>10
80	2.5	5	>10
	4.2	9	>10
85	2.5	2	1
	4.2	6	10
90	2.5	2	2
	4.2	1	1
95	2.5	1	1
	4.2	1	1
100	2.5	1	1
	4.2	1	1

after six weeks did not show any further change except for a slight clarification in all cases where the pH was 4.5. The degree of clarification in each lot at different temperatures and intervals remained unaltered.

After storage for six months marked differences were observable, and these increased after 12 months. The apparent degree of clarification of all samples after storage for six and 12 months is shown (Tables 3 to 5).

Since the decomposition of pectic substances in the previous experiments by Joslyn and Sedky (1940) was always accompanied by clarification of the juice, the clarification of the juice on standing could be taken as a sign of the existence and activity of these en-

TABLE 4

Effect of Heating at pH 2.5 and 4 on Clearing of Grapefruit Juice

Temperature of bath	pH	Heating to prevent clearing at various temperatures	
		After 6 months	After 12 months
°C.		min.	min.
60	2.5	>10	>10
	4.0	>10	>10
65	2.5	>10	>10
	4.0	>10	>10
70	2.5	>10	>10
	4.0	>10	>10
75	2.5	7	>10
	4.0	>10	>10
80	2.5	2	3
	4.0	5	6
85	2.5	1	1
	4.0	1	1
90	2.5	1	1
	4.0	1	1
95	2.5	1	1
	4.0	1	1
100	2.5	1	1
	4.0	1	1

TABLE 5

Effect of Heating at pH 2.2 and 4 on Clarification of Lemon Juice

Temperature of bath	pH	at various temperatures at various temperatures	
		After 6 months	After 12 months
°C.		min.	min.
60	2.2	>10	>10
	4.0	>10	>10
65	2.2	>10	>10
	4.0	>10	>10
70	2.2	1	>10
	4.0	>10	>10
75	2.2	1	5
	4.0	2	5
80	2.2	1	1
	4.0	1	1
85	2.2	1	1
	4.0	1	1
90	2.2	1	1
	4.0	1	1
95	2.2	1	1
	4.0	1	1
100	2.2	1	1
	4.0	1	1

zymes. Where the juice remains turbid, it is inferred that the enzymes were rendered inactive by heating.

For both orange juice and grapefruit juice at pH 4 the inactivation temperature for a heating period of one minute was 80°C.

At their natural pH. Heated for minutes shown.

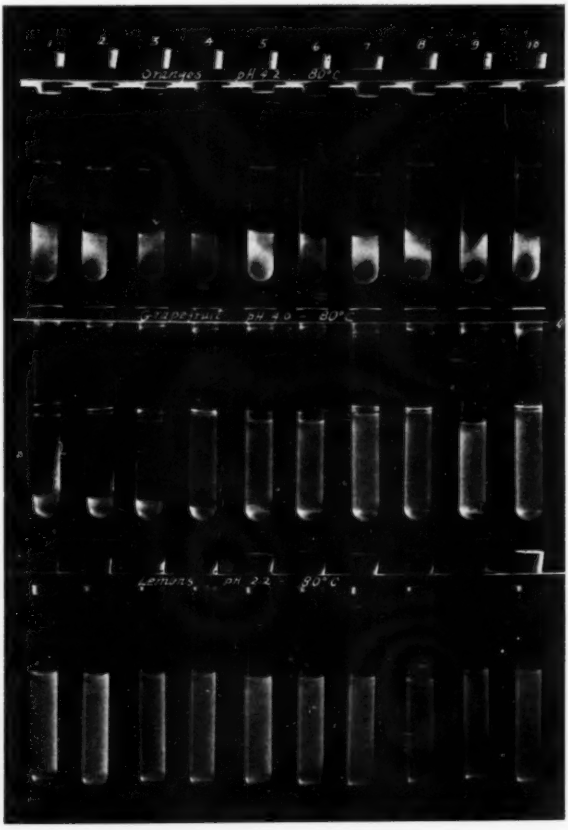

FIG. 1. A comparison of the effect of relative heat inactivation of the clearing pectic enzymes in oranges, grapefruit, and lemons.

(176°F.), while that of lemons was 75°C.(167°F.). At pH 2.5 the inactivation temperature for both orange juice and grapefruit juice is 75°C. and that of lemon juice is 70°C.(158°F.). From this it is concluded that in lemon juice at either pH range, the pectic enzymes were inactivated at a lower temperature than in either orange juice

or grapefruit juice at corresponding pH values. It is also concluded that at higher pH value, a higher temperature is required to inactivate the enzyme, or a longer time is required at the same temperature.

Typical results are shown in three photographs taken of suitably arranged samples (Figs. 1, 2, and 3). The samples for each fruit

At the higher pH range. Heated for minutes shown.

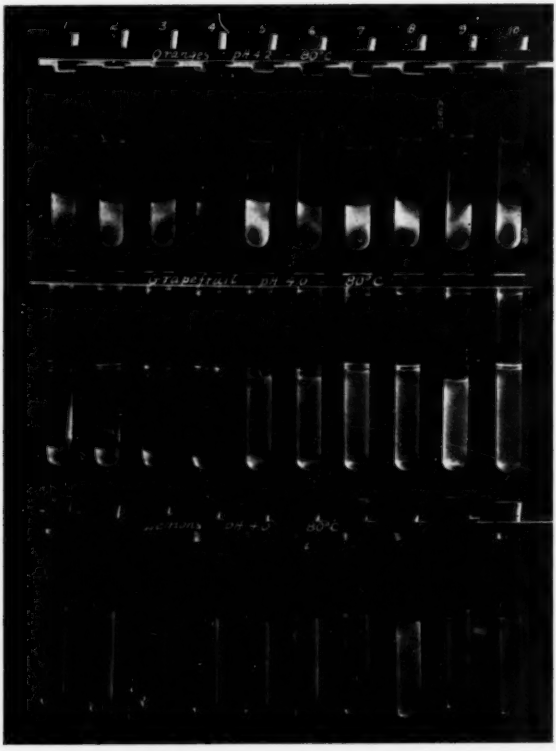

FIG. 2. A comparison of the effect of relative heat inactivation of the clearing pectic enzymes in oranges, grapefruit, and lemons.

were arranged from left to right according to the length of heating period from one to 10 minutes. Samples arranged in this way for all the fruits were compared. By comparing them at a constant temperature, it is possible to observe the inactivation of the pectic enzymes for all fruits at their natural pH values, at a constant pH of 2.5, and at a constant pH of 4.

Since 80°C. was in general close to the inactivation temperature, it was employed as the constant so that the variations with pH and type of fruit would be clearly shown.

In a comparison of the activity of the enzymes in the three fruits at their natural pH (Fig. 1) it is apparent that these enzymes are

At the lower pH range. Heated for minutes shown.

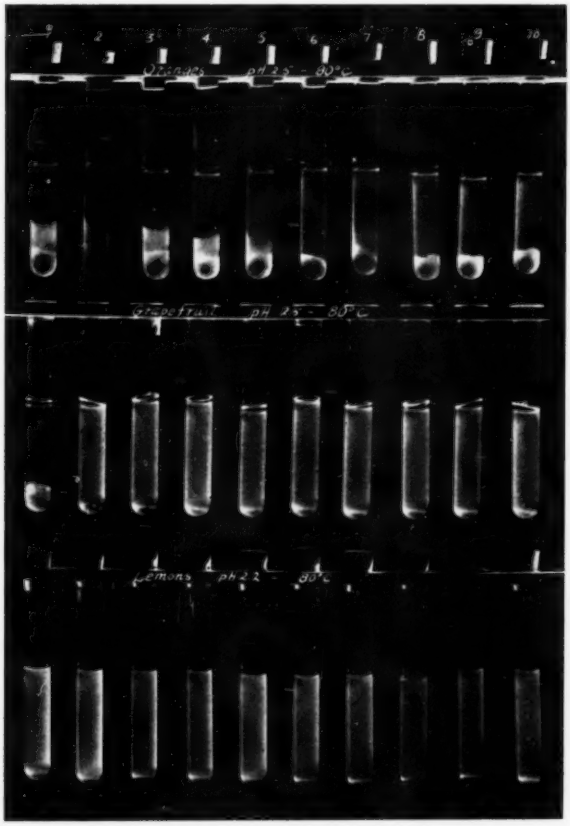

FIG. 3. A comparison of the effect of relative heat inactivation of the clearing pectic enzymes in oranges, grapefruit, and lemons.

more active in orange juice than in grapefruit and least active in lemon juice. The latter fact is partly due to the low natural pH value, at which the enzymes are less active.

The same comparison is shown at a constant pH of 4 (Fig. 2); the only difference between this and the previous comparison is that

the pH value of lemon juice has been raised to that of the other two fruits. This caused an increase in the enzymatic activity in it.

The accomplishment of the same purpose by lowering the pH values of orange and grapefruit juice to that of lemon (Fig. 3) caused a decrease in the enzyme activity for both oranges and grapefruit.

At pH 4 the enzymes in orange and grapefruit juice are rendered inactive at 80°C. but they remained active at 75°C.

In lemons there was no instance of complete inactivation in a range between 70 and 80°C., though at a temperature lower than 80°C., the enzymes were more active.

At constant temperature for each separate fruit the pectic enzymes were found to be more active at pH 4 than at pH 2.2, and more heat resistant.

The extent of inactivation owing to higher acidity is greater than that owing to variation in temperature.

SUMMARY

The effect of pH, temperature, and time of heating on the inactivation of enzymes responsible for the clearing of citrus juices was investigated. It was found that clearing of juices occurred at lower rates the higher the temperature or the longer the time of heating; that the enzymes in oranges and grapefruit were somewhat more heat resistant than those in lemons; that the enzymes in Valencia oranges were more heat resistant than those in navel oranges; that the heat inactivation was more rapid at the lower of the two pH values studied.

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VARIATIONS IN ASCORBIC ACID CONTENT OF GRAPEFRUIT AND ORANGES FROM THE RIO GRANDE VALLEY OF TEXAS

EMILY METCALFE, PEGGY REHM,[1] AND JET WINTERS
Home Economics Department, University of Texas, Austin, Texas

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Since the introduction of chemical methods for determination of the ascorbic acid content of foods and the finding that the indophenol dye titration method is particularly suited to citrus fruit juices, many investigations of the ascorbic acid content of citrus fruit have been undertaken. In addition to data on California, Florida, and Texas fruit, material is available on fruit grown in such widely separated countries as China, India, Palestine, South Africa, and British Honduras. Efforts have been made to correlate variation in ascorbic acid content with difference in variety, season, location, and acid content of fruit. The effects of sterilization, storage, and spraying on ascorbic acid content have also been investigated. The present investigation is concerned with the ascorbic acid content of oranges and grapefruit grown in the Rio Grande Valley of Texas.

REVIEW OF LITERATURE

Throughout this report ascorbic acid content will be expressed in milligrams per 100 cubic centimeters of juice, and figures referring to ascorbic acid content should be interpreted on this basis. Hawley (1937), as a result of a large number of determinations made on a wide range of variety and at different seasons, gave the average ascorbic acid content of orange juice as 50 and of grapefruit juice as 40. Daniels and Rutherford (1937), using eight varieties of oranges, found a variation of 32 to 62. Richardson, Davis, and Sullivan (1937), using two varieties of California oranges, obtained 20 to 30; but Daniels, Kennedy, and Munsell (1936), for the same two varieties, reported 40 to 58. For Florida oranges Daniels, Kennedy, and Munsell (1936) reported variation of 45 to 51; while Becham and Bonner (1937), working with six varieties of Florida oranges, obtained 45 to 57. Floyd and Fraps (1939) assayed 217 various fruits and vegetables grown in Texas in 1937. Among these were grapefruit (six varieties) which varied from 31 to 44, and oranges (six varieties) which varied from 29 to 46. Palestine oranges, according to Yofe and Yofe (1938), varied from 32 to 62;

[1] Died January 28, 1940.

those from China, Su, Liu, and Tu (1939), from 9 to 44; and South African oranges, Hamersma (1939), from 38 to 83. Oranges from India, according to Wats and White (1931), have approximately one-fifth the ascorbic acid content of western oranges. This figure was based on biological evaluations. The suggestion was made that the variation might be due to difference in species since the India oranges belong to the Nagpur rather than the Malta species, the latter being the species found in England and America.

Not a great deal of material is available on grapefruit. Daniels and Rutherford (1937) state that the ascorbic acid content of grapefruit approaches the lower limits of that of oranges, i.e., about 35. Bracewell and Zilva (1931), comparing grapefruit from South Africa and British Honduras with Jaffa oranges from Palestine, found the grapefruit had a higher ascorbic acid content than the oranges. This is not in line with the reports of other investigators on the relative ascorbic acid content of oranges and grapefruit. Two varieties of Florida grapefruit showed variations of 37 to 38, Becham and Bonner (1937). It would seem that the average of 40 suggested by Hawley is somewhat high.

Small but consistent differences in ascorbic acid content of different varieties of oranges and grapefruit have been reported. Pineapple oranges were about 10 per cent higher than Valencia oranges according to two reports on Florida fruit by Daniels, Kennedy, and Munsell (1936) and Becham and Bonner (1937). In these same reports California navel oranges and Florida Temple oranges are shown to be unusually high in ascorbic acid content.

Various investigators reported loss of ascorbic acid content as the citrus fruit season advances. Palestine oranges, Yofe and Yofe (1938), were about ten per cent lower at the end than at the beginning of the season. Two varieties of oranges grown in South Africa, Hamersma (1939), showed a decrease in ascorbic acid content as the season advanced but other varieties remained constant. Abbott and French (1937) reported, for Florida oranges, that ascorbic acid decreased to and beyond maturity in late ripening oranges but that with early and mid-season oranges, an increase in ascorbic acid preceded the subsequent decrease. Becham and Bonner's (1937) figures on Florida fruit also showed small decreases toward the end of the season.

There seems to be little evidence for consistent variation with location, i.e., in no investigation reported were all varieties of fruit from one place of higher ascorbic acid content than similar varieties from other locations. It is true, however, that the same variety of fruit grown in different localities varied considerably in ascorbic acid

content. In the Florida investigations by Becham and Bonner (1937) Valencia oranges from 13 different locations varied from 36 to 55, while pineapple oranges from nine different places varied from 46 to 67.

English investigators, Bacharach, Cook, and Smith (1934), reported that single oranges from different sources varied from 22 to 89. Hamersma (1939), in South Africa, reported that there were significant differences in the ascorbic acid content of fruit from trees of the same variety in the same orchard and from orchards in different localities, the last difference being greater than the first. He states that these differences were correlated with differences in the acidity of the juices. Abbott and French (1937), working with Florida fruit, also reported a correlation between total acid and ascorbic acid content of juice, but Bacharach *et al.* (1934) found no correlation between the pH of juice and ascorbic acid content.

Several investigators have reported no loss in ascorbic acid content of citrus fruit when stored for two months at 15°C.(59°F.), but others have reported losses as high as 20 per cent. Processing of oranges to destroy Mediterranean fruit fly larvae consists of heating them for eight hours at 43.3°C.(110°F.). Nelson and Mottern (1932) reported that this did not affect ascorbic acid content. The same investigators found that spraying with lead arsenate brought about 33 to 50 per cent loss of ascorbic acid.

EXPERIMENTAL PROCEDURE

The data on Texas fruit were obtained on oranges and grapefruit furnished by six different growers [2] in the Rio Grande Valley, two representing the eastern, two the central, and two the western part of the section. Samples were sent at the beginning, middle, and end of the citrus fruit season. All six growers sent samples for the beginning of the season, five sent samples for the middle, but only four at the end. Requests were made for Marsh, Duncan, and Foster grapefruit and for Valencia, Hamlin, and Temple oranges. Since there are four different kinds of Marsh grapefruit—plain Marsh, Marsh seedless, Marsh pink, and Improved Pink or Redblush—it was decided to treat each as a separate variety. In addition to the oranges selected for analysis, a few samples of navel, Joppa, and pineapple were sent, and data on these are included. The citrus fruit season lasts approximately from the middle of November until

[2] Samples were obtained from the following locations: Pitt Brothers Citrus Nurseries, Brownsville, Texas; Engleman Gardens, Edcouch, Texas; Bay View Citrus Association, Los Fresnos, Texas; Mission Citrus Association, Mission, Texas; Stuart Place Citrus Association, Harlingen, Texas; Sam J. Baker and Son, San Benito, Texas.

the middle of April but each variety of orange has its own and a shorter season. Navels and Hamlin are available early, but Valencias and Temples do not reach maturity until January. Determinations for oranges were obtained only for the beginning and end of the season. The first determinations were run late in November, the second in late February or early March, and the third late in April. Fruit was stored at 7.2°C.(45°F.) immediately upon arrival, and determinations were always completed within a week. Preliminary work had shown that no change in ascorbic acid content occurred in this time.

The indophenol dye [3] was standardized according to the improved method of Menaker and Guerrant (1938), in which potassium iodide and sulphuric acid are added to a dye solution of known concentration. This mixture is then titrated against N/100 sodium thiosul-

TABLE 1

Variation in Ascorbic Acid Content of Different Varieties of Grapefruit and Oranges [1, 2]

Grapefruit		Oranges	
Marsh	36.4	Navel	41.1
Marsh Pink	32.4	Valencia	42.3
Red Blush	32.7	Hamlin	50.0
Marsh Seedless	32.0	Joppa	46.9
Foster Pink	34.3	Pineapple	54.2
Duncan	37.7	Temple	52.4

[1] Average of all determinations regardless of location or season. [2] Figures based on milligrams of ascorbic acid per 100 c.c. of juice.

phate and adjusted so that 100 c.c. of the dye solution is equivalent to one milligram of ascorbic acid. (One c.c. of N/100 iodine solution is equivalent to .88 mgm. ascorbic acid.) The dye solution was also titrated against pure ascorbic acid and the two results agreed closely. Fresh solutions of the dye were prepared every two days. Distilled water, redistilled from glass, was used throughout the work. Titration of fruit juice against iodine, according to the method of Stevens (1938), gave results which were almost identical with those obtained with dye.

To prepare the fruit juice for titration a mixed sample from four grapefruit or six oranges was used. Filtering the juice through two thicknesses of cheesecloth proved just as satisfactory a method of clearing as the longer method of centrifuging. Diluted grapefruit juice was titrated without the addition of acid since it did not change in ascorbic acid content over a short period of time. Orange juice,

[3] Hoffman-LaRoche, Nutley, N. J.

however, gave a sharper end point when acetic and metaphosphoric acid were added, and the addition of the acid was necessary to stabilize the ascorbic acid content.

DISCUSSION OF RESULTS

The ascorbic acid contents of the different varieties of grapefruit and oranges are shown as averages of all determinations regardless

TABLE 2

Seasonal Variations in Ascorbic Acid Content of Grapefruit and Oranges[1]

	GRAPEFRUIT					
Time	Marsh	Marsh Pink	Red Blush	Marsh Seedless	Foster Pink	Duncan
Nov.	39.2	35.5	36.6	.....	44.9	41.3
Feb.	34.7	33.5	31.8	34.0	32.3	36.4
April	27.9	28.5	24.9	30.0	29.5	33.0

	ORANGES	
	Valencia	Hamlins
Nov.	.....	51.6
Feb.	42.5	46.0
April	38.2	.....

[1] Figures based on milligrams of ascorbic acid per 100 c.c. of juice.

of location or season (Table 1). The number of samples analyzed varied from eight to 12 for grapefruit and six to eight for oranges (except navel, Temple, and Joppa on which only a few determinations were run). As will be seen by comparing these figures (Table 1)

TABLE 3

Comparison of Texas Fruit With Florida and California Fruit[1]

	Florida	California	Texas
Pineapple oranges	52	....	55
Valencia oranges	47	40	42
Navel oranges	....	58	41
Temple oranges	57	....	52
Hamlin oranges	46	....	50
Joppa oranges	45	....	47
Marsh Seedless grapefruit	38	....	32
Foster Pink grapefruit	37	....	34

[1] Figures based on milligrams of ascorbic acid per 100 c.c. of juice.

with those showing seasonal variation and variation with location (Tables 2 and 4, respectively) different varieties of grapefruit and oranges varied less in ascorbic acid content than the same variety

at different times in the season, or the same variety grown in different locations. The decrease in ascorbic acid content as the season advanced was quite marked and is greater than that reported by Becham and Bonner (1937) for Florida fruit. These experiments were carried through April, however, while the Florida investigations were terminated in March. Variations with location, presumably due primarily to soil differences, were quite inconsistent. No grower produced samples of different varieties all of which were higher than similar samples submitted by other growers. Moreover,

TABLE 4

Variation in Ascorbic Acid Content of Grapefruit and Oranges From Different Locations[1, 2]

Grapefruit					
Location	Marsh	Marsh Pink	Red Blush	Foster Pink	Duncan
No. 1	43.8	.....	40.7	43.0	.....
No. 2	39.0	.....	38.0	.....	45.3
No. 3	36.0	37.8	.....	46.9	38.4
No. 4	40.7	35.8	.....	34.8	48.7
No. 5	32.1	35.8	34.8	35.3	36.4
No. 6	43.3	32.6	33.0	35.0	38.0

Oranges				
Location	Navel	Valencia	Hamlin	Pineapple
No. 1	44.8	.....	.....	.....
No. 2	.....	44.6	44.4	.....
No. 3	36.6	42.1	.....	62.3
No. 4	41.9	41.0	43.1	47.3
No. 5	.....	40.0	47.9	54.8
No. 6	.....	41.3	47.7	.....

[1] Figures based on milligrams of ascorbic acid per 100 c.c. of juice. [2] Figures from first shipment only, since not all growers sent all three shipments.

first, second, and third samples from the same grower did not always hold the same rank in respect to samples from other growers. Grapefruit juice seems to vary less than orange juice in ascorbic acid content, but it is always definitely lower. Comparative figures for Texas, California, and Florida fruit indicate little difference in the ascorbic acid content of fruit from these three locations (Table 3).

In order to determine whether ascorbic acid content was affected by cold storage, part of the fruit from one shipment was placed in an ordinary cold storage vault for one month. Titration of juice from the stored fruit showed no decrease in ascorbic acid content. From one grower it was possible to get samples of both untreated and processed fruit. No difference in the ascorbic acid content of the two samples was detected.

SUMMARY

Ascorbic acid content of grapefruit and oranges grown in the Rio Grande Valley section of Texas was determined by the indophenol titration method. Seasonal variation as well as variation in different varieties and according to location were investigated. Results show rather small variation in the different varieties tested, considerable but inconsistent variation on the basis of location, and a very definite reduction in ascorbic acid content at the end of the season. The ascorbic acid content of Texas citrus fruit was closely comparable to that of Florida and California fruit. Samples kept in cold storage for one month showed no decrease in ascorbic acid. Processing of fruit to destroy the Mediterranean fruit fly larvae did not affect ascorbic acid content.

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VARIETAL DIFFERENCES IN ASCORBIC ACID (VITAMIN C) CONTENT OF STRAWBERRIES[1]

G. HOWARD SATTERFIELD
Department of Chemistry, University of North Carolina, Raleigh, North Carolina

AND

MARY YARBROUGH
Department of Chemistry, Meredith College, Raleigh, North Carolina

(Received for publication, September 5, 1939)

Until recently only scattered reports have appeared in the literature dealing with the ascorbic acid (vitamin C) content of strawberries *(Fragaria virginiana)*.

Nearly 20 years ago, Smith, Bergeim, and Hawk (1921) reported that 10 ml. of strawberry juice daily cured guinea pigs of scurvy in seven days. Kohman, Eddy, and Halliday (1928) found that two to three grams of strawberries daily served to protect guinea pigs. Strawberries were shown by von Hahn (1931) to be an extremely good source of ascorbic acid. Strawberry juice assayed by MacLeod (1935) had 80 units per pound.

Chinese strawberries were reported by Chi and Read (1935) to contain 49 to 69 mg. per 100 grams, and by Chang and Collier (1936) 50 mg. per 100 grams. Fernandez and Alfageme (1936) showed that Spanish strawberries had 46 mg. of ascorbic acid per 100 grams. In France, Giroud, Ratsimamanga, Leblond, Chalopin, and Rabinowicz (1936) found that strawberries contained 66 mg. ascorbic acid per 100 grams. The ascorbic acid content of Dutch strawberries was reported by van Eekelen (1938), after treatment with mercuric acetate and hydrogen sulfide, to be 68 mg. per 100 grams. American grown strawberries studied by Bessey (1938) with the Evelyn photoelectric colorimeter contained 52 mg. per 100 grams; Kirk and Tressler (1939) found the juice by visual titration to have .495 mg. per ml. and when determined by the titrimeter to have .489 mg. per ml.

Several workers have reported on the ascorbic acid content of definite varieties of strawberries. Fellers and Mack (1933) found that two grams daily of either the Howard Supreme or the Klondike gave good growth and protected guinea pigs from scurvy. One and one-half grams daily of the Saxon were shown by Bukin, Povolotzkaya, and Onokova (1934) to prevent scurvy in guinea pigs. Levy

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and Fox (1935) stated that the Royal Sovereign contained 52 mg. of ascorbic acid per 100 grams. These results were confirmed by Charley (1936), who gives the following figures: Royal Sovereign, 52.9; Oberschlesein, 64; and Tardive de Leopold, 40 mg. per 100 grams. On the other hand, Olliver (1938) found the following ascorbic acid content in milligrams per 100 grams: Royal Sovereign, 69; Jucunda, 62; Paxton, 61; Evern, 55; and Brenda Gautrey, 55.

In view of these reports of other workers, the varietal differences in the ascorbic acid content of American strawberries seemed worthy of investigation.

EXPERIMENTAL PROCEDURE

Through the courtesy of Professors M. E. Gardner, E. B. Morrow, and J. G. Weaver of the Department of Horticulture, School of Agriculture, University of North Carolina, seven varieties of strawberries grown under identical conditions of soil, fertilizer, and climate were secured. The samples for analysis were carefully selected and only firm, ripe fruit of characteristic size for each variety was used.

The method used for the determination of ascorbic acid was essentially that described by Bessey and King (1933) and modified by Musulin and King (1936) and Mack and Tressler (1937). Five to eight berries weighing about 25 grams were ground with acid-washed sand and extracted with two 30-ml. portions of an acid mixture containing eight per cent acetic acid and four per cent metaphosphoric acid. The extracts were centrifuged and decanted into a separatory funnel. The pigment was partially removed, as suggested by McHenry and Graham (1935), by shaking the extract with 75 ml. of butyl alcohol, drawing off the aqueous portion and washing the alcohol portion with 15 ml. of distilled water. The extract and the washing from the alcohol were made up to 100 ml. with the acid mixture. A 2-ml. aliquot was pipetted into a No. 3, Diamond I capsule vial and titrated with 2, 6-dichlorophenolindophenol (25 mg. in 100 ml.), using a modified Neale-Forbes Titration Assembly according to the technique of Satterfield, Perlzweig, and Dann (1936). The yellow-pink extract was titrated to a blue-pink end point. The dye was standardized against pure ascorbic acid.

Since the completion of this investigation, Evelyn, Malloy, and Rosen (1938) and Bessey (1938) have published methods for the determination of ascorbic acid using the Evelyn photoelectric colorimeter; Kirk and Tressler (1939) used an electrometric titrimeter to assay strawberries.

DISCUSSION

The ascorbic acid contents of seven varieties of strawberries grown during the 1938 season (Table 1) show a definite varietal difference.

It may be observed that the average ascorbic acid content of the Fairmore is 79 per cent greater than the average of the Missionary and that the smallest quantity found in the Fairmore is 24 per cent greater than the largest amount in the Missionary. We offer no explanation of the noticeable variation within the varieties. Olliver (1938) has shown that strawberries from the same plant have a high initial concentration of ascorbic acid, and as the season progresses there is a gradual decline followed by an upward trend. Our data are not such that a comparable analysis may be made.

It should be interesting to note that the two varieties with the lowest ascorbic acid content are standard commercial varieties that

TABLE 1

Varietal Differences in Ascorbic Acid (Vitamin C) Content of Strawberries

Variety						
Fairmore	Fairfax	Southland	Dorsett	Blakemore	Klondike	Missionary
mg./gm.	*mg./gm.*	*mg./gm.*	*mg./gm.*	*mg./gm.*	*mg./gm.*	*mg./gm.*
.738	.645	.530	.492	.533	.415	.481
.672	.584	.511	.491	.484	.412	.429
.670	.583	.506	.471	.468	.402	.402
.661	.562	.505	.471	.462	.397	.401
.647	.546	.496	.469	.448	.394	.342
.645	.545	.465	.450	.389	.393	.329
.640	.545	.463	.447	.388	.387	.329
.612	.517	.453	.427	.374	.383	.312
.602	.497	.....	.413	.355	.360	.300
.593	.475	.....	.397	.354	.339	.294
.648	.550	.491	.453	.426	.388	.362

are widely planted. According to Darrow and Waldo (1935) the Klondike originated about 1896 and the Missionary in 1900. On the other hand, the highest ascorbic acid content was found in the Fairmore, a new berry, which was originated in 1933 as a cross between the Fairfax and the Blakemore and was introduced in 1939 by Darrow and Morrow (1939).

The Fairmore is vigorous, is noted for its firmness and shipping quality, is uniform in shape, is deep red in color, and possesses high dessert quality.

If, as King (1936) suggests, the daily human requirements for ascorbic acid are 40 mg., this need may be met by eating 20 to 30 strawberries.

The ascorbic acid content of the seven varieties of strawberries in this study, varying from .294 to .738 mg. per gram, compares

favorably with that of the seven varieties of oranges that Olliver (1936) found to range from .189 to .769 mg. per milliter of juice.

SUMMARY

Varietal differences in the ascorbic acid content of seven varieties of strawberries produced under the same conditions are shown.

The average ascorbic acid content of the Fairmore is 79 per cent greater than the average for the Missionary.

Twenty to 30 strawberries daily will meet the human needs for ascorbic acid.

Strawberries are equal to oranges in ascorbic acid content.

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ASCORBIC ACID CONTENT OF CABBAGE AS INFLUENCED BY VARIETY, SEASON, AND SOIL FERTILITY

R. C. BURRELL, H. D. BROWN, AND VIRGINIA R. EBRIGHT
Department of Agricultural Chemistry and Department of Horticulture, The Ohio State University, Columbus, Ohio

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The more important literature dealing with the ascorbic acid content of foods, which appeared prior to 1936, has been reviewed by Daniel and Munsell (1937) who have tabulated the values reported by the various investigators. For uncooked cabbage, the quantity of ascorbic acid per gram of fresh weight varied from .133 to 1 milligram, with slightly higher values in one instance for certain leaves of the head. There is thus almost an eight-fold difference in recorded values. Undoubtedly the method of analysis has influenced some of the results as much as actual differences in ascorbic acid content. Until satisfactory means had been devised to prevent the rapid oxidative loss of ascorbic acid from the plant extracts tested, not much reliance could be placed in the values determined by chemical methods. By the use of metaphosphoric acid, as employed by Fujita and Iwatake (1935) and Musulin and King (1936), and high acidity, Mack and Tressler (1937), the greater part of such loss can be eliminated.

Even when recently determined values obtained by approved methods are compared, however, considerable variation in the quantity of ascorbic acid in cabbage is observed. Gould and Tressler (1936) report the ascorbic acid contents of six varieties of cabbage. The results in milligrams per gram of fresh weight were as follows: Golden Acre, .55; Early Copenhagen, .55; Peerless, .56; Late Copenhagen Market, .26; Glory, .30; and Danish Ballhead, .30. Varieties maturing early in the summer are higher in vitamin C content than the later varieties which are grown for kraut and storage. Floyd and Fraps (1939) found 1 to 1.58 milligrams of ascorbic acid per gram of fresh weight in several small-headed varieties of cabbage. Varietal variations in cabbage have also been reported recently by certain Russian investigators whose papers, however, are generally available in abstract form only.

EXPERIMENTAL PROCEDURE

It was thought interesting to investigate further to what extent certain external and internal factors can alter the ascorbic acid

content of cabbage. Specifically, it was planned: (1) to study possible varietal differences in ascorbic acid content, not only of standard varieties, but also of certain new varieties and strains which are being developed in the plant-breeding program of the University and the Ohio Agricultural Experiment Station; (2) to determine whether any such differences in ascorbic acid content persist unchanged from season to season; and (3) to start work on a project which should deal with the effect of various fertilizers on the ascorbic acid content of various vegetables, including cabbage.

Thirty-one varieties and strains of cabbage grown under similar conditions in the University gardens at Columbus, Ohio, were tested for ascorbic acid content. A sprinkler system made sufficient moisture available at all times during growth. Analyses were made immediately after harvesting or not later than following over-night storage in a refrigerator at 4°C.(39.2°F.).

Two heads of each variety were used. Quarters were cut from each head, the cores removed, and the leaves coarsely chopped with a knife as quickly as possible. Twenty-gram samples were placed immediately in mortars which contained 30 c.c. of one normal sulfuric acid which was also two per cent in metaphosphoric acid. The samples were ground with about 10 grams of acid-washed sand until they were thoroughly disintegrated. The more fluid portion from each was then transferred to a large centrifuge tube and centrifuged. The clear liquid was poured into a 100 c.c. graduated flask; the residue in the mortar was further ground with about 20 c.c. of the acid mixture; and again the liquid was poured into the same centrifuge tube. The caked residue in the bottom of the tube was broken up with a glass rod and centrifuging repeated. The clear supernatant liquid was added to the portion already in the graduated flask. Once again, or until tests showed no more dye decolorizing material being extracted, the grinding and centrifuging were repeated. The combined extracts from each sample were made up to volume with distilled water; and 10-c.c. aliquots, diluted to 50 c.c. with eight per cent acetic acid if desired, were titrated against 2,6 dichlorophenol-indophenol indicator solution (the "dye solution") which had been previously standardized the same day against pure vitamin C. The end point was the first faint pink color that persisted for 15 seconds. This is essentially the same method that has been successfully used by Tressler (1938) and his associates. The sulfuric-metaphosphoric acid solution must be freshly prepared. Even keeping it over night leads to lower vitamin results than otherwise. The dye solution may be kept for several days, but must be restandardized each day against pure vitamin C (ascorbic acid) solution.

TABLE 1

Ascorbic Acid Content of 30 Varieties and Strains of Cabbage in Relation to Average Weight per Head, Earliness, and Total Yield From 100 Plants

Variety	Seed grower [1]	Ascorbic acid per gram of fresh weight	Average weight per head	Harvested before July 6	Total yield
		mgm.	*lb.*	*pct.*	*lb.*
Allhead Select	N.K.P.A.	0.480	2.48	34.32	236.00
Wisconsin Y.R. No. 8	F.	0.499	1.76	22.48	175.75
Ohio Station No. 5[2]	O.A.E.S.	0.546	1.46	99.22	128.25
Allhead	A.S.G.	0.563	2.17	16.59	153.75
Ohio Station No. 7[2]	O.A.E.S.	0.605	1.70	82.80	134.75
Detroit Resistant	F.	0.607	1.69	78.53	146.75
Wisconsin Hollander	A.H.	0.622	1.78	2.37	126.50
Ohio Station No. 12[2]	O.A.E.S.	0.628	1.22	85.38	106.00
Ohio Station No. 10[2]	O.A.E.S.	0.677	1.24	96.64	119.00
Ohio Station No. 11[2]	O.A.E.S.	0.692	1.36	93.80	121.00
Early Wonder	S.G.	0.703	1.46	96.39	124.25
Ohio Station No. 2[2]	O.A.E.S.	0.711	1.12	91.86	95.25
Copenhagen Market	F.	0.719	1.46	75.23	111.00
Marion Market Resistant	S.G.	0.813	2.07	54.06	197.00
Golden Acre	A.H.	0.825	1.55	83.90	118.00
Allseasons	F.	0.829	2.14	46.56	192.75
Golden Acre, Y.R.	H.	1.025	1.44	95.63	126.00
Penn State Ballhead	F.	1.067	1.76	34.95	138.75
Charleston	A.S.G.	1.075	1.89	79.42	173.75
Hollander	F.	1.085	1.72	49.40	144.75
Early Dwarf Drumhead	S.G.	1.162	1.19	97.27	109.75
Danish Ballhead	S.	1.270	1.70	42.88	130.00
Green Acre	H.	1.370	1.79	73.04	159.50
Marion Market	F.	1.416	2.15	75.10	186.75
Babyhead Special	S.G.	1.480	1.11	100.00	94.00
Early Select, No. 99	H.	1.651	1.64	87.33	154.00
Ohio Station No. 8[2]	O.A.E.S.	1.699	1.39	90.98	122.00
Golden Acre Special	H.	1.710	1.64	87.33	154.00
Wisconsin Allseasons, Y.R.	F.	1.728	2.23	50.30	203.25
Midseason Market	F.	1.809	2.15	66.33	172.25
Average for above 30 varieties and strains	.....	1.002	1.68	69.70	145.16
Average for 10 highest in ascorbic acid	.....	1.529	1.70	77.06	148.55
Average for 10 lowest in ascorbic acid	.....	0.592	1.69	61.21	144.78

[1] A.H. = A. Hansen Co.; A.S.G. = Associated Seed Growers; F. = Ferry-Morse Seed Co.; H. = The Holmes Seed Co.; N.K.P.A. = National Kraut Packers Association; O.A.E.S. = Ohio Ag'l Expt. Station; S. = E. Suhr Co.; S.G. = Sluiss en Groot, Enkhuizen, Holland.
[2] Unnamed strains of Golden Acre.

This chemical method for vitamin C determination has been found to check very satisfactorily with the vitamin C potency as determined by biological assay by Tressler, Mack, and King (1936).

RESULTS AND DISCUSSION

The results of the analyses dealing with varietal differences are expressed (Table 1); the results for the varieties which were tested

TABLE 2

Seasonal Variations in Ascorbic Acid Content of Cabbage

Date analyzed	Variety	Ascorbic acid per gram of fresh weight
		mgm.
7-13-38	Ohio Station No. 5 [1]	0.546
11- 3-38	Ohio Station No. 5	0.502
7-14-38	Ohio Station No. 7	0.605
11- 3-38	Ohio Station No. 7	0.608
7-15-38	Ohio Station No. 11	0.692
11-10-38	Ohio Station No. 11	0.692
7-13-38	Ohio Station No. 12	0.628
11-10-38	Ohio Station No. 12	0.729
7-20-38	Ohio Station No. 2	0.711
11- 8-38	Ohio Station No. 2	0.771
7-14-38	Ohio Station No. 10	0.677
11-10-38	Ohio Station No. 10	0.861
7-15-38	Ohio Station No. 8	1.699
11- 8-38	Ohio Station No. 8	1.004
7-28-38	Savoy Chieftain	1.162
10-27-38	Savoy Chieftain	1.068
7-25-38	Detroit Resistant	0.607
8- 1-39	Detroit Resistant	0.695
7-26-38	Allseasons	0.829
8- 1-39	Allseasons	0.738
6-28-38	Midseason Market	1.809
8- 1-39	Midseason Market	1.021

[1] Ohio Station varieties are unnamed strains of Golden Acre.

at different seasons, e.g., summer and autumn, and a few which were analyzed for two successive years, are set forth (Table 2); and finally a preliminary report of tests of the same variety grown under different fertilizer treatments is made (Table 3).

The values given in the tables represent the results of analyses of duplicates from two separate samples which were taken from each of two heads of the same variety, i.e., an average of eight separate titrations. It will be noted that values ranged from .480 milligrams of ascorbic acid per gram for Allhead Select to 1.809 for

Midseason Market (Table 1). Good agreement in results of analyses was obtained between separate samples from the same head. Between separate heads of the same variety, the average deviation in ascorbic acid content for 50 such pairs was 7.27 per cent, with a range of zero to 17 per cent.

In the fertilizer studies the Allseasons variety was grown on plots of Miami silty clay loam and watered as required by means of a sprinkler system. To other similar plots various quantities of nitrogen (as sulfate of ammonia), potassium (as muriate of potash), and phosphorus (as superphosphate) were applied; the exact quantities

TABLE 3

Effect of Fertilizer Treatment on Ascorbic Acid Content of Allseasons Cabbage

Plot No.	Fertilizer treatment (pounds per acre)			Ascorbic acid per gram of fresh weight (two separate heads)	
	Sulfate of ammonia 21% N	Muriate of potash 50% K_2O	Superphosphate 20% P_2O_5		
				mgm.	*mgm.*
1	.....	.....	.....	.723	.730
2	.....	240	1,000	.701	.702
3	381	240	.....	.816	.819
4	381	.....	1,000	.799	.819
5	381	240	1,000	.864	.824
6	762	240	1,000	.899	.889
7	381	240	2,000	.803	.801
8	381	480	1,000	.768	.793

are indicated (Table 3). Exceptionally good agreement was obtained between heads from the same treatment. However, since the work here reported on varietal differences (Tables 1 and 2) shows some variation in the quantity of ascorbic acid present in different heads of the same variety, more reliable data would be obtained if a larger number of heads—probably a dozen from each plot—were sampled. It is proposed to continue the present investigation for several years. Thus far it would seem that high nitrogen or a complete fertilizer are most effective in increasing the ascorbic acid content of cabbage.

An attempt was made to relate average weight per head, earliness, or total yield to ascorbic acid content (Table 1). Earliness, i.e., the per cent harvested before July 6, seems to be the only factor which is correlated with high ascorbic acid. Yield, average weight per head, and amount harvested before July 6 are all based on results from 100 plants of each variety or strain.

SUMMARY

For the 31 varieties and strains of cabbage tested in this investigation, the ascorbic acid content varied between .480 and 1.809 milligrams per gram of fresh weight.

The same variety did not have numerically the same ascorbic acid content at different seasons or in different years, although, in general, varieties that are high in ascorbic acid as compared with other varieties, tend to remain so; and those that are low remain low.

Preliminary tests in fertilizer experiments, indicate that high nitrogen or a complete fertilizer gives rise to a higher ascorbic acid content in cabbage than do six other treatments.

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VITAMIN B_1 CONTENT OF EIGHT VARIETIES OF BEANS GROWN IN TWO LOCALITIES IN MICHIGAN[1]

EUNICE KELLY, KATHLEEN S. DIETRICH, AND THELMA PORTER
Department of Foods and Nutrition, Division of Home Economics, Michigan State College, East Lansing, Michigan

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Michigan holds first place among the states in the acreage devoted to field beans as well as in the total production of this crop. The field-bean crop of Michigan, according to the annual crop summary for 1938, occupied nearly one-half million acres, and had a total value of more than eight and one-half million dollars. This money value represents about 11 per cent of the value of all field crops in this state for 1938, Crop Report of Michigan (1939).

Michigan produces several kinds of beans, including a large percentage of the total United States' crop of the pea-bean, red kidney, and cranberry varieties. It is generally recognized that dry beans are economical sources of a number of dietary essentials including energy, body-building proteins, and certain minerals and vitamins.

The purpose of this study was the determination of the vitamin B_1 content of eight varieties of beans grown in two localities of Michigan.[2]

EXPERIMENTAL PROCEDURE

Albino rats from 25 to 29 days old, weighing from 48 to 57 grams were used as experimental animals in this study. The young animals were taken from the stock colony and placed in individual cages with raised screen floors, to prevent access to excreta. They were fed the vitamin B_1-deficient diet of Chase and Sherman (1931). It consisted of vitamin B_1-free casein, 18 per cent; butterfat, eight per cent; starch, 53 per cent; Osborne and Mendel salt mixture, four per cent; autoclaved yeast, 15 per cent; and cod liver oil, two per cent. At the end of the first week the animals were weighed and then put into harnesses to further guard against coprophagy. Beginning on the 10th or 11th day of this depletion period the animals were weighed on alternate days until loss of weight or cessation of growth indicated that their vitamin B_1 stores were exhausted.

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[2] The authors wish to acknowledge with sincere appreciation the help of Assistant Professor H. R. Pettigrove, Farm Crops Department, Division of Agriculture, in procuring all samples of beans tested in this investigation.

One animal from each litter was used for either a negative or a positive control, an even distribution of the sexes being made in these groups. Negative control animals remained on the basal diet until death. Positive control animals were divided into two groups. One group received, for five weeks, graduated levels of the International Standard Adsorption Product of vitamin B_1 which was diluted with cornstarch, one part of the adsorbate being mixed with 49 parts of cornstarch. Quantities of this dry mixture equivalent to 5, 10, or 15 milligrams of the adsorbate were fed daily in small supplement dishes. These quantities of the International Standard represent .5, 1.0, and 1.5 International Units of vitamin B_1, respectively. The second group of positive control animals was fed graduated amounts of crystalline vitamin B_1.[3] A stock solution of the crystals was made up in 25 per cent alcohol; and this solution, diluted with distilled water, was administered from small supplement dishes in one-, two-, and three-milliliter volumes, equivalent to two, four, and six micrograms of the crystals, respectively.

Average weekly weight increments of groups of rats on each level of these two reference materials served as the basis for the construction of curves of response used for the interpretation of data on the beans. A curve was constructed from the equation $y = a + b$ (log x) with y representing the average weight changes in grams per week and x, the quantities of reference material fed daily, Coward (1938) and Miller (1939).

Since a control animal was selected from each litter, the growth responses of these animals correspond exactly in season and in experimental conditions to those of the test animals. Forty-four animals served as negative controls and 138 animals as positive controls.

All remaining animals in the litters, as equally distributed as to sex and weight as possible, were given graduated levels of dry raw ground beans to be assayed for their thiamin content. The eight varieties of beans *(Phaseolus vulgaris)* grown in two localities in Michigan included the Blue Pod, Robust, Great Northern, 1200-1, Kidney, Yellow Eye, Cranberry, and a newly developed variety called Michelite by Down and Thayer (1938). All varieties of beans were procured from the Fowlerville district, Livingston county; and all but the new Michelite from the locality around Romeo, Macomb county.[4]

[3] Betabion, Crystalline Vitamin B_1 Merck.

[4] The soil in the Fowlerville area was a light to medium sandy loam; that in the Romeo district, a heavy sandy loam rather rolling in character. On the farms where these beans were raised no special fertilizer applications were made. The operation of farms in these communities, however, includes a general sequence of cropping as usually practiced in this area northwest of Detroit.

All of the samples, except the Michelite beans, were from the 1936 crop. The assays were begun in the spring of 1937, and continued in the spring and summer of 1938. In the spring of 1938, the samples tested the previous spring were re-assayed and no change in vitamin value was found. It was therefore considered that the storage of the samples in a dry, cool place was not influencing the vitamin content of the beans. The first seven varieties listed from the Fowlerville district were fed at the three levels of .25, .50, and .75 gram daily (seven days a week). The Michelite was given at the .5-gram level only. The varieties from Romeo were fed at these three levels also,

TABLE 1

Data on Positive and Negative Control Rats

Daily supplement	Quantity fed daily	Number of rats	Average length of depletion period	Average food consumption per week	Average weight at depletion	Average weekly change in weight
Negative controls	*mg.* 0	44	*days* 14.8	*gm.* 20.9[1]	*gm.* 69.4	*gm.* —7.9 ± .4[2]
Crystalline vitamin B_1	0.002	20	14.7	32.1	67.3	1.2 ± .4
	0.004	20	14.6	40.8	67.7	6.0 ± .5
	0.006	20	15.0	41.9	67.0	7.2 ± .5
Vitamin B_1 International Reference Standard (adsorbate)	5.0	28	13.9	29.6	70.6	—0.6 ± .3
	10.0	30	15.5	35.0	71.0	5.2 ± .4
	15.0	20	14.4	39.4	68.0	7.7 ± .5

[1] Survival time was 21.5 days. [2] Arithmetic mean ± standard error.

except for the 1200-1 and Cranberry varieties which were given in daily doses of .5 gram only.

The daily weighed supplements of beans were usually fed in separate dishes, but in some instances, when difficulty in administering the raw ground beans was encountered, the weighed amounts were mixed with a small quantity of the basal diet in order to induce the animals to eat the supplement. Animals failing to consume all of the daily supplements were not included in the calculation of growth responses. A total of 460 animals were fed bean supplements. In addition to weekly weights of the animals, weekly food consumptions were recorded throughout the experiment. On all series of animals the mean growth responses with their standard deviations and standard errors were calculated.

RESULTS AND DISCUSSION

The data on the response of control rats to crystalline thiamin hydrochloride and to the International adsorbate (Table 1) show that

these groups of animals averaged from 67 to 71 grams in weight at the end of the depletion period, which was usually about two weeks long. Average weekly food consumption increased with the administration of increasing quantities of the reference materials. Animals receiving daily two, four, and six micrograms of thiamin hydrochloride showed mean weight gains of 1.2, 6.0, and 7.2 grams per week, respectively. These gains are represented by cross marks (x) at the proper points (Fig. 1). Groups of animals fed daily 5, 10, and 15 milligrams of the standard adsorption product exhibited

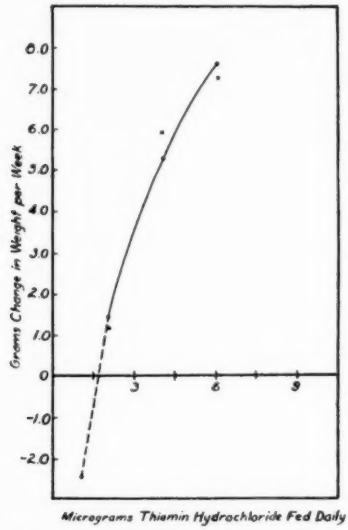

FIG. 1. Calculated curve of response of groups of rats to doses of crystalline thiamin hydrochloride. The crosses indicate the actual mean gains in weight per week of groups of rats fed two, four, or six micrograms of the crystals daily.

weight changes of —0.6, 5.2, and 7.7 grams per week, as shown by cross marks (Fig. 2). Negative control animals showed an average weekly loss of 7.9 grams. They survived an average of 21.5 days. These average weight changes are similar to those obtained in other studies of the rats from this colony when kept in harnesses on a thiamin-deficient diet. The number of animals and the length of the experimental period warrant the use of the data for the construction of curves of reference, Sherwood and Halverson (1938) and Miller (1939). The equation representing the relationship between the quantities of crystalline vitamin B_1 fed and the mean gains in weight per week was found to be $y = -2.48 + 12.96 (\log x)$. The curve cor-

responding to gains in weight on the International adsorption product is drawn from the equation $y = -12.8 + 17.7 (\log x)$.

An inspection of the two graphs drawn to the same scale reveals a difference in the slope of the two lines. The curve representing the response of animals to graduated levels of crystalline thiamin hydrochloride is less steep and therefore covers a wider range of vitamin values than that based on response to the adsorbate.

The data on the response of animals fed graduated quantities of dry ground beans as a supplement to the vitamin B_1-deficient diet are given (Tables 2 and 3). The average weight of the animals at

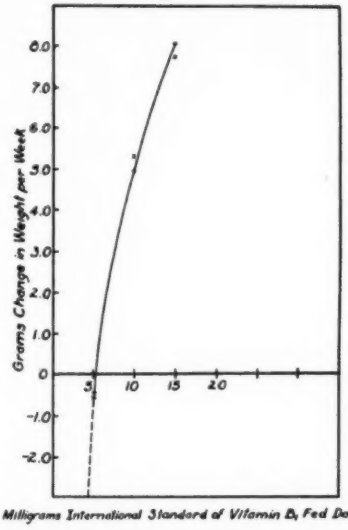

FIG. 2. Calculated curve of response of groups of rats to doses of International Standard Adsorption Product. The crosses indicate the actual mean gains in weight per week of groups of rats fed 5, 10, or 15 milligrams of the adsorbate daily.

the time the diet was supplemented with the beans was 70 grams (range 64 to 77 grams); the average length of the depletion period was 13 to 15 days. Thus, the experimental animals were as uniformly depleted in vitamin B_1 as their litter mates, the control animals.

Average weekly food consumption (Tables 2 and 3) ranged from 22 to 40 grams. Invariably an increase in the level of beans from .25 to .50 gram fed daily was accompanied by an increase in the total food consumption, this increase varying from two to 14 grams per week. With the next increase in the size of the bean supplement, however, there was not always a corresponding increase in food con-

sumption; for instance, in groups of animals fed beans from Romeo (Table 3) the average weekly food consumption on the .75-gram bean supplement was usually less than it was on a .5-gram bean supplement. Difficulty was met in successfully administering the .75-gram level of

TABLE 2

Data on Rats Fed Ground Raw Beans Grown Around Fowlerville, Livingston County, Michigan, 1936

Variety of *Phaseolus vulgaris*	Quantity fed daily	Number of rats	Average length of depletion period	Average weekly food consumption	Average weight at depletion	Average weekly change in weight
	gm.		*days*	*gm.*	*gm.*	*gm.*
Blue Pod	.25	12	14.6	31.4	63.9	2.0 ± .6[1]
	.50	12	14.5	38.6	65.3	5.8 ± .6
	.75	12	14.8	40.2	68.9	7.4 ± .7
Robust	.25	12	13.8	31.1	70.3	0.7 ± .6
	.50	15	14.8	38.0	70.7	5.1 ± .6
	.75	12	14.0	36.1	67.8	6.1 ± .8
Great Northern	.25	10	14.0	28.3	70.5	—1.2 ± .8
	.50	14	15.2	35.3	71.5	4.1 ± .7
	.75	10	14.2	36.0	70.8	3.7 ± .9
1200-1	.25	12	14.1	29.4	70.3	0.2 ± .4
	.50	12	14.7	34.9	70.8	4.3 ± .8
	.75	12	14.8	34.8	75.3	4.7 ± .9
Kidney	.25	12	14.4	26.2	70.3	—1.7 ± .6
	.50	11	14.2	31.8	77.6	2.6 ± .6
	.75	11	15.1	29.9	71.2	2.0 ± .9
Yellow Eye	.25	11	13.7	23.3	71.3	—2.7 ± .5
	.50	11	14.3	26.8	67.8	0.2 ± .8
	.75	11	14.3	25.9	67.9	0.2 ± .8
Cranberry	.25	12	13.8	22.7	66.3	—2.4 ± .6
	.50	12	15.1	27.9	70.7	0.7 ± .6
	.75	11	13.5	24.9	67.3	0.4 ± .8
Michelite	.50	10	13.2	31.1	67.5	3.6 ± .7

[1] Arithmetic mean ± standard error.

beans. The animals apparently were in some way adversely affected by the high ingestion levels. The result was that total basal food consumptions were not as high as might have been expected on the .75-gram level of beans.

The effects of "leveling off" of the food consumption were reflected in the average weekly gains of the animals. In several instances the lowest quantity of a given variety of bean fed furnished so little available vitamin B_1 that little gain or even a loss in weight was exhibited. As the quantity of beans used to supplement the diet increased from .25 to .50 gram there was always an increment in the average weekly gain in weight. This was not true,

TABLE 3

Data on Rats Fed Ground Raw Beans Grown Around Romeo, Macomb County, Michigan, 1936

Variety of *Phaseolus vulgaris*	Quantity fed daily	Number of rats	Average length of depletion period	Average weekly food consumption	Average weight at depletion	Average weekly change in weight
	gm.		*days*	*gm.*	*gm.*	*gm.*
Blue Pod	.25	12	13.8	26.4	70.1	—0.3 ± .6[1]
	.50	16	15.4	40.1	72.0	7.7 ± .6
	.75	12	14.4	32.0	69.0	6.1 ± .6
Robust	.25	12	13.8	28.8	69.6	1.8 ± .6
	.50	13	13.8	32.6	70.5	5.1 ± .6
	.75	12	14.2	34.8	72.0	5.4 ± .8
Great Northern	.25	12	14.3	24.9	67.4	—0.7 ± .6
	.50	12	14.0	29.4	65.8	3.1 ± .6
	.75	12	13.5	28.9	70.2	2.2 ± .5
1200-1	.50	12	14.9	28.2	67.8	1.6 ± .6
Kidney	.25	12	13.1	26.8	74.0	—2.4 ± .6
	.50	12	13.3	29.3	71.6	1.9 ± .6
	.75	12	14.0	29.0	71.8	1.1 ± .8
Yellow Eye	.25	9	14.9	22.6	67.4	—1.8 ± .5
	.50	11	14.1	26.3	68.5	0.4 ± .6
	.75	10	14.5	25.9	69.2	1.0 ± .8
Cranberry	.50	12	14.8	24.0	66.7	—0.1 ± .5

[1] Arithmetic mean ± standard error.

however, for the next "step-up" in the quantity of beans fed. In as many as half of the varieties of beans, the animals fed .75-gram levels gave a lower average weekly gain in weight than those on the .5-gram levels.

VITAMIN B_1 VALUES ESTIMATED FROM CURVE OF RESPONSE TO CRYSTALLINE THIAMIN HYDROCHLORIDE

The vitamin B_1 content of the eight varieties of beans assayed are given (Table 4); the vitamin potencies are expressed in Inter-

national Units per gram of raw ground bean as estimated from both curves of response (Figs. 1 and 2).

Each vitamin value represents an average of three readings corresponding to the three levels at which the beans were fed. The readings from the curves were converted to the one-gram basis in each case, then averaged, and transferred to International Units. Three micrograms of crystalline thiamin hydrochloride, or 10 milligrams of the International Standard Adsorption Product, were considered equivalent to one International Unit.

The results based on estimations from the curve of response to thiamin hydrochloride crystals were as follows: The vitamin B_1 values range from approximately three International Units to one International Unit per gram. In both localities the Blue Pod beans ranked first and the Robust beans second in vitamin B_1 content. The Blue Pods contained 2.9 (Fowlerville) and 2.7 (Romeo) International Units per gram and the Robust, 2.4 units (both localities). The Great Northern beans showed a vitamin B_1 content of slightly over 1.5 International Units per gram and ranked third among the varieties grown in Romeo and fifth among those from Fowlerville. The 1200-1 variety from Fowlerville had about two International Units of vitamin B_1 per gram, while the Romeo-grown sample of this variety contained approximately 1.5 units. Kidney beans from both districts ranked fifth in their vitamin B_1 content, which ranged from 1.2 to 1.4 units per gram. The Cranberry and Yellow Eye beans contained approximately one International Unit per gram from both localities. The new variety, Michelite, contained about two International Units per gram, ranking third in vitamin value among the beans grown in Fowlerville and comparing favorably with the 1200-1.

Of the seven varieties of beans obtained from both communities, four of the Fowlerville-grown varieties show slightly higher vitamin B_1 values, two show the same value, and one shows less vitamin B_1 than corresponding varieties grown around Romeo. These differences are probably insignificant, except in the case of the 1200-1 variety which shows a difference of .6 unit per gram in favor of the Fowlerville product.

COMPARISON OF VITAMIN VALUES ESTIMATED FROM BOTH CURVES OF RESPONSE

The vitamin values read from the curve of response of rats to the International Adsorption Product are not identical with those from the curve based on thiamin feeding. Nevertheless, they are of the same order of magnitude and would permit the same general interpretation of the findings. The comparative ratings between varieties and between the two communities are the same read from either curve.

If a given growth response, such as three grams per week, is converted to International Units, a comparison can be made. Three grams of growth per week resulted from 2.7 micrograms of thiamin, or .9 International Unit; the same weekly growth on the other curve was exhibited with the feeding of 8.25 milligrams of the adsorbate, or .825 International unit. These differences are not real discrepancies but probably represent expected differences in the responses of animals to these two reference products. A comparison of vitamin B_1 potencies of a given variety of bean as read from the two curves seems to indicate that the crystalline thiamin is readily and com-

TABLE 4

Vitamin B_1 Content of Beans (Phaseolus vulgaris) as Estimated From Curves of Response of Rats to Crystalline Thiamin Hydrochloride and to International Reference Adsorbate in International Units[1]

Variety	International Units of vitamin B_1 per gram of beans			
	From curve of response to thiamin hydrochloride		From curve of response to International adsorbate	
	Fowlerville	Romeo	Fowlerville	Romeo
Blue Pod	2.9	2.7	2.4	2.2
Robust	2.4	2.4	2.0	2.1
Great Northern	1.7	1.6	1.6	1.5
1200-1	2.0	1.4	1.8	1.4
Kidney	1.4	1.2	1.5	1.3
Yellow Eye	1.0	1.1	1.1	1.3
Cranberry	1.1	1.1	1.2	1.1
Michelite	2.0	.....	1.8	.....

[1] One International Unit is equivalent to three micrograms of thiamin.

pletely available at all levels fed, and that the adsorbate is probably less readily assimilated when fed in the large doses. A study of the curves along with the values (Table 4) shows that for varieties of beans of relatively high vitamin value, slightly higher values are read from the curve of response to crystalline thiamin hydrochloride than from the "adsorbate" curve. The values read from the lower portions of the curves, while not identical, are more nearly alike. As was indicated in the earlier reference to the general directions of the two curves, the vitamin B_1 potencies estimated from the "thiamin" curve cover a slightly larger range than those determined by the International Standard. The difference between the highest and lowest vitamin values read from the curve of response to thiamin hydrochloride is 1.9 units (2.9—1.0); that from the "adsorbate" curve is 1.3 units (2.4—1.1).

The results of these assays on the vitamin B_1 content of beans indicate that they are a very good source of the vitamin. Some vari-

eties are nearly three times as rich in this essential nutrient as others; but even the varieties with the lowest values are comparable with whole wheat, certain nuts, and egg yolk in vitamin B_1.

SUMMARY

The vitamin B_1 content of eight varieties of beans *(Phaseolus vulgaris)* grown in two localities in Michigan was determined by the rat-growth method. Forty-four animals served as negative controls, 138 as positive controls, and 460 as experimental animals.

A curve of response was constructed from the average growth of three groups of 20 animals each fed respectively two, four, and six micrograms of crystalline thiamin hydrochloride.

Estimations of the vitamin B_1 content of the different varieties of beans indicated that the values ranged from about three International Units to one Unit per gram.

The Blue Pod and Robust varieties were highest in vitamin B_1 content; the Cranberry and Yellow Eye were lowest. Great Northern, 1200-1, Kidney, and Michelite were of medium value as sources of vitamin B_1.

There were no generally significant differences in the vitamin values of beans grown in the two communities in Michigan, although a number of values were slightly higher in Fowlerville beans than in Romeo beans.

Vitamin potencies estimated from a curve of response based on graduated levels of crystalline thiamin hydrochloride are not identical with those read from a curve based on the International Adsorption Product. The comparative ratings of the vitamin values of the different kinds of beans, however, are the same if estimated from either curve of response.

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INFLUENCE OF AGE OF COW ON ASCORBIC ACID CONTENT OF CERTIFIED MILK

ARTHUR D. HOLMES AND FRANCIS TRIPP
Research Laboratories, The E. L. Patch Company, Boston, Massachusetts
AND
ELMER A. WOELFFER
H. P. Hood & Sons, Boston, Massachusetts
AND
G. HOWARD SATTERFIELD
University of North Carolina, Raleigh, North Carolina

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Numerous investigations have been conducted to determine the ascorbic acid content of milk. Studies have been made of the relation between ascorbic acid content of the ration and that of the milk produced from it by the following: Hess, Unger, and Supplee (1920); Hart, Steenbock, and Ellis (1920); Dutcher, Eckles, Dahle, Mead, and Schaefer (1920); Olson and Copeland (1924); Hughes, Fitch, Cave, and Riddell (1927); MacLeod (1927); Rasmussen, Geurrant, Shaw, Welch, and Bechdel (1936); Riddell, Whitnah, Hughes, and Lienhardt (1936); Reedman (1937); Hannisdahl (1937); and Whitnah and Riddell (1937). Some investigators—Rasmussen, *et al.* (1936), Whitnah and Riddell (1937), and Holmes, Tripp, Woelffer, and Satterfield (1939b)—have compared the ascorbic acid content of the milk produced by common breeds of dairy cows, and others—Whitnah and Riddell (1937); Holmes, Tripp, Woelffer, and Satterfield (1939); de Wildt, de Ruijter, and Brouwer (1930); and Schwartze, Murphy, and Hann (1930)—have determined the vitamin C content of milk produced at different seasons of the year. A score or more of investigators—Olson and Copeland (1924); Reedman (1937); Schwartze, Murphy, and Cox (1931); Conn, Johnson, Trebler, and Karpenko (1935); King and Waugh (1934); Riddell and Whitnah (1936); Tauber (1936); Sharp (1936); Kon and Watson (1936); Whitnah, Riddell, and Caulfield (1936); Hand, Guthrie, and Sharp (1938); Dann and Satterfield (1937); and Holmes, Tripp, Woelffer, and Satterfield (1939a)—have accumulated considerable data concerning the effect of the pasteurization of milk upon its ascorbic acid content. Recent reports—Ingalls (1938); Hawley (1938); and West and Wenger (1938)—contain results of studies conducted to determine the vitamin C content of milk when it reaches the ultimate consumer. A

survey of the literature reveals little information concerning any relationship between the age of the cow and the ascorbic acid content of her milk; this study was conducted to obtain such data.

EXPERIMENTAL WORK

Mature, purebred, and high-grade Guernsey and Holstein cows kept under carefully controlled conditions for 12 months were used in the study. The individual animals were selected so that they represented a significant range in age and different stages of pregnancy and lactation. The Guernseys varied from four to 11 years and the Holsteins from five to nine years, inclusive. The cows were stall fed and allowed outdoors only for exercise. These cows, which were a part of a herd of 175 cows, were maintained under as nearly as possible ideal conditions for the production of certified milk.

In a few instances when the milk flow of a cow materially diminished owing to the end of the lactation period or when the amount of milk produced became so small as to make the cow unprofitable, she was removed from the experiment and replaced by an animal, comparable except for the diminished milk flow, that had been maintained in the certified herd under conditions similar to those of the experimental animals.

A total of 28 Guernsey and 27 Holstein cows was used in this experiment.

MEDICAL AND VETERINARY INSPECTION

Since the experimental animals were maintained as a part of a herd producing certified milk, all the attendants were under constant medical supervision to eliminate human infections or milk-borne diseases.

The animals are accredited to the tuberculin test and negative to Bangs Disease (infectious abortion). Mastitis was controlled by a continuation of a series of barn and laboratory tests together with approved sanitary practices. Clinical examinations and classification of the udders by an experienced veterinarian constituted an important part in the mastitis-control program. Wendt (1938) claims that the vitamin C content of cows' milk depends greatly upon the condition of the udder; Rasmussen, Guerrant, Shaw, Welch, and Bechdel (1936) state that milk from cows afflicted with mastitis has abnormal ascorbic acid value.

Obviously, careful attention was given to keeping the cows in continuous, excellent physical condition and producing a profitable yield of milk.

RATIONS

In planning the experimental ration for the cows an attempt was made to maintain uniform conditions throughout the 12-months period. All cows received a daily ration compounded from various

TABLE 1

Composition of Dairy and Fitting Rations

Dairy Ration	
	pct.
Calcium carbonate	0.5
Cane molasses	5.0
Corn gluten feed	10.0
Cottonseed meal, 41% (hydrolic process 5% fat)	10.0
Distillers' corn grains dried	10.0
Iodized salt	1.0
Oats (crimped)	17.5
Soy bean oil meal (expeller process)	5.0
Steamed bone meal	1.0
Wheat bran	25.0
Yellow corn meal	5.0
Yellow hominy feed	10.0
Home-Mixed Fitting Ration	
Calcium carbonate	1.0
Cane molasses	7.0
Iodized salt	1.0
Oats (crimped)	30.0
Soy bean oil meal (expeller process)	10.0
Steamed bone meal	1.0
Wheat bran	25.0
Yellow corn meal	10.0
Yellow hominy feed	15.0
Commercial Fitting Ration	
Alfalfa meal	Unknown
Calcium carbonate	Unknown
Carotene	Unknown
Corn gluten meal	Unknown
Corn meal	Unknown
Crushed oats	Unknown
Dried beet pulp	Unknown
Iodized salt	Unknown
Molasses	Unknown
Soy bean oil meal	Unknown
Wheat bran	Unknown
Wheat middlings	Unknown

combinations of the following ingredients: first and second grades of alfalfa hay, first and second grades of mixed hay, legume and grass silage, beet pulp, dairy ration, dairy ration with 6.7 per cent

irradiated yeast, home-mixed fitting ration with 6.7 per cent irradiated yeast, and a commercial fitting ration with irradiated yeast. Obviously, it was not possible at the beginning of the experiment to assemble and store all the concentrates and roughage to be consumed during the 12-months period. Accordingly, from time to time it was necessary to purchase new lots of alfalfa hay. Also at intervals during the year, when a mow of the hay which was grown locally had been consumed, it was necessary to begin feeding a fresh supply. The same was true for the silage. As the contents of one silo were exhausted it was necessary to begin to draw upon another

TABLE 2

Ascorbic Acid Content of Components of Experimental Ration

	Ascorbic acid per 100 gm. of feed
	mg.
Beet pulp	0.669
Commercial fitting ration with irradiated yeast	1.622
Dairy ration	0.562
Dairy ration with irradiated yeast	0.754
Ensilage (grass)	1.548
Fitting ration and irradiated yeast	0.592
Hay, alfalfa, first grade, baled	1.883
Hay, alfalfa, second grade, baled	1.380
Hay, good farm, one-half alfalfa	1.334
Hay, second grade, all grass	0.907

silo. However, these changes from lot to lot of grain or roughage did not affect the uniformity of the rations. The composition of the various concentrates is reported in detail (Table 1).

ASSAY METHOD

Samples of the concentrates and the different kinds of hay were assayed for their ascorbic acid content by Tillman's method as modified by Bessey and King (1933). Five-gram samples were ground with 30 grams of acid-washed sand; 25 to 35 c.c. of an acid mixture containing four per cent metaphosphoric and eight per cent acetic acid were added, as suggested by Musulin and King (1936). The mass was centrifuged, the supernatant liquid was decanted, and the residue was repeatedly extracted until 50 c.c. of extract were obtained. Five cubic centimeters of the extract, diluted to 25 c.c., were titrated with 2,6-dichlorophenolindophenol as described by Tripp, Satterfield, and Holmes (1937). The results of these assays are reported (Table 2).

The samples of milk, which were assayed for their vitamin C content, were taken at bimonthly intervals at the morning milking (3:00 a.m.). The samples were immediately cooled, placed on ice, and protected from the light until they were assayed. The assays were made between six and 12 hours after the samples of milk were obtained from the cows. During the 52-weeks experimental period 322 samples of Guernsey milk and 337 samples of Holstein milk were assayed for their vitamin C content. The amount of ascorbic acid in the milk was determined by the indophenol-titration method. Five cubic centimeters of milk were added to a 2.5-c.c. mixture of eight per cent acetic acid and four per cent metaphosphoric acid in a 50-c.c. Erlenmeyer flask. The dye solution was added from a microburette drop by drop until a faint pink end point was reached which remained for 30 seconds. The strength of the dye solution was equivalent to .08 to .10 mg. of vitamin C per cubic centimeter. This solution was prepared and standardized in a manner similar to that described by Tripp, Satterfield, and Holmes (1937).

RESULTS

The results of assays of the milk produced by the Guernsey cows have been grouped according to the ages of the cows producing the milk (Table 3). The data reported consist of the maximum, minimum, and average ascorbic acid contents for each group of cows at each assay period. The final column of the table contains information concerning the total number of cows which were included in each group during the 12-months period, and the average ascorbic acid content for the entire number of assays of each group. Similar data for the Holstein cows are shown (Table 4).

As was to be expected, it was impossible to provide an equal number of cows in all the age groups considered. The herd from which the experimental animals were selected contained very few cows under six years of age and few animals over nine years of age. However, results obtained with four Guernsey cows, 4, 5, 10, and 11 years old respectively, have been included as a matter of interest.

The smallest amount of ascorbic acid found in the milk from the six-year-old Guernsey cows (Table 3) was 12.32 mg. per 1,000 c.c. on Sept. 30. The largest amount of ascorbic acid produced by these cows was 27.4 mg. per 1,000 c.c. on Feb. 17. The average amount of ascorbic acid varied from 17.1 mg. on Sept. 30 to 23.6 mg. on Feb. 17. The values for the seven-year-old Guernsey cows were minimum 17.5 mg. on May 6, and maximum 31.4 mg. on Oct. 14, the lowest average being 19.9 mg. on May 6 and the highest average

TABLE 3

Ascorbic Acid Content of Milk[1] as Influenced by Age of Cow—Guernsey

Age		Aug. 19	Sept. 16	Sept. 30	Oct. 14	Oct. 28	Nov. 10	Nov. 24	Dec. 9	Dec. 23	Jan. 6	Jan. 20	Feb. 3	Feb. 17	March 3	March 23	April 7	April 21	May 6	May 19	June 2	June 23	July 8	July 21	Aug. 11	Group av.
yr.																										
4	No. of cows...	.....	.....	.....	.....	.....	.....	.....	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	.....	.....	19.40
	Min.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	15 assays
	Max.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1 cow
	Av.....	.....	.....	.....	.....	.....	.....	.....	20.20	19.50	20.80	19.70	21.10	19.10	20.60	20.31	18.93	18.70	15.71	19.70	18.64	19.54	17.90	.....	.....	
5	No. of cows...	.....	.....	.....	.....	.....	1	1	.....	.....	1	1	1	1	1	1	1	1	1	1	1	1	.....	1	.....	19.00
	Min.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	15 assays
	Max.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1 cow
	Av.....	.....	.....	.....	.....	.....	21.01	21.50	.....	.....	20.80	21.20	22.13	22.42	19.82	18.80	15.60	18.70	14.70	17.02	17.61	17.83	.....	15.50	.....	
6	No. of cows...	3	6	6	6	7	6	6	6	5	4	4	4	4	5	5	4	4	5	4	5	5	5	5	5	20.41
	Min.....	16.20	17.60	12.32	19.21	17.90	18.70	17.00	19.90	16.24	18.52	19.00	18.63	19.51	18.30	15.00	15.22	18.40	15.40	19.40	17.61	17.53	15.90	15.50	19.31	119 assays
	Max.....	20.03	22.10	20.80	23.13	23.04	24.30	22.80	23.44	20.80	23.72	27.00	25.30	27.40	24.02	23.00	22.30	22.30	19.90	23.70	22.10	23.70	22.93	21.30	25.50	11 cows
	Av.....	18.20	20.10	17.10	21.34	20.70	21.34	20.60	21.10	18.71	21.21	22.10	21.62	23.60	21.04	20.24	19.60	20.20	17.90	21.53	20.20	20.60	20.84	18.12	22.84	
7	No. of cows...	4	5	5	4	4	4	4	4	5	5	5	5	5	4	4	5	5	4	4	3	3	4	4	3	21.93
	Min.....	17.70	19.04	17.70	20.40	20.80	21.50	20.90	20.84	18.53	19.50	21.20	19.70	21.60	20.60	19.20	17.82	18.40	17.50	18.40	19.00	20.60	20.60	19.10	22.00	102 assays
	Max.....	24.63	29.81	25.80	31.40	27.50	25.70	27.03	27.70	22.74	23.40	27.00	26.00	28.23	25.20	23.80	23.02	22.00	21.70	24.03	21.80	22.00	22.60	22.14	25.03	9 cows
	Av.....	21.00	22.00	21.10	23.63	23.60	23.30	23.13	23.12	20.53	21.60	23.42	22.14	24.20	22.70	21.40	20.80	20.53	19.91	21.00	20.71	21.30	21.90	20.50	24.00	
8	No. of cows...	2	2	2	2	2	2	2	2	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	19.53
	Min.....	17.30	17.24	16.20	19.61	20.80	21.01	20.20	21.16	.....	18.52	20.42	21.10	22.42	19.82	18.40	18.20	13.80	14.00	19.40	17.61	19.90	16.90	17.30	.....	46 assays
	Max.....	18.80	18.70	20.02	20.60	21.60	23.82	21.82	22.14	.....	22.73	23.71	22.13	23.30	19.82	20.31	18.60	18.40	16.41	19.40	19.33	19.90	18.60	17.72	.....	4 cows
	Av.....	18.01	18.00	18.10	20.10	21.20	22.42	21.01	21.70	18.53	20.63	22.10	21.62	22.84	19.82	19.40	18.40	16.10	15.20	19.40	18.50	19.90	17.71	17.50	21.10	
10	No. of cows...	1	1	1	1	1	1	1	1	1	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	18.70
	Min.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	9 assays
	Max.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1 cow
	Av.....	19.50	17.60	16.94	20.00	16.74	20.60	20.20	19.54	17.23	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
11	No. of cows...	.....	.....	.....	.....	.....	.....	.....	.....	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	20.11
	Min.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	16 assays
	Max.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	1 cow
	Av.....	.....	.....	.....	.....	.....	.....	.....	.....	19.83	22.10	25.90	23.20	24.91	18.70	19.94	19.31	18.04	19.90	19.40	16.91	17.83	18.90	16.83	21.10	

[1] Milligrams per 1,000 c.c. of milk

24.2 mg. on Feb. 17. Similar values for the eight-year-old cows were minimum 13.8 mg. on April 21, and maximum 23.8 mg. on Nov. 10, the lowest average being 15.2 mg. on May 6 and the highest average 22.8 mg. on Feb. 17. The yearly averages were for 119 assays of milk from 11 six-year-old Guernsey cows, 20.4 mg. per 1,000 c.c. of milk; for 102 assays of milk from nine seven-year-old cows, 21.9 mg.; and for 46 assays of milk from four eight-year-old cows, 19.5 mg. The average values for the individual 4, 5, 10, and 11-year-old Guernsey cows were 19.4, 19.0, 18.7, and 20.1 mg., respectively, per 1,000 c.c. of milk. These data seem to indicate that there is no consistent relationship between the age of Guernsey cows and the ascorbic acid content of their milk.

The distribution of the Holstein cows through the five age groups was more uniform than that for the Guernsey cows. The least amount of ascorbic acid found in the milk from the five-year-old Holstein cows (Table 4) was 9.8 mg. per 1,000 c.c. on April 21. The greatest amount of ascorbic acid was 19.4 mg. on May 19. The average amount of ascorbic acid varied from 11.0 mg. on June 23 to 17.7 mg. on Dec. 9. The minimum and maximum values for the six-year-old Holstein cows were 11.5 mg. of ascorbic acid per 1,000 c.c. on March 23 and 24.9 mg. on Feb. 17. The lowest average for the group was 16.5 mg. on April 21, and the highest average was 20.9 mg. on Dec. 9. The values for the seven-year-old cows were minimum ascorbic acid 15.6 mg. per 1,000 c.c. of milk on Dec. 23, and maximum 23.2 mg. on Feb. 17. The average values for the group varied from 16.4 mg. on April 21 to 23.0 mg. on Feb. 17. The minimum amount of ascorbic acid for the eight-year-old group of Holstein cows was 14.1 mg. for Aug. 11, and the maximum was 22.4 mg. on Feb. 17. The average ascorbic acid for the group varied from 15.4 mg. on May 6 to 23.1 mg. on Nov. 10. The ascorbic acid content of milk produced by the group of nine-year-old Holstein cows was minimum 11.7 mg. on May 19, maximum 25.3 mg. on Oct. 28, and the average values varied from 15.0 mg. on May 19 to 19.4 mg. on Dec. 9. The yearly averages were for 30 determinations of milk from four five-year-old Holstein cows, 15.3 mg. per 1,000 c.c. of milk; for 125 determinations of milk from 10 six-year-old cows, 18.6 mg.; for 36 determinations of milk from three seven-year-old cows, 18.9 mg.; for 55 determinations of milk from four eight-year-old cows, 19.1 mg.; and for 91 determinations of milk from six nine-year-old Holstein cows, 17.6 mg. per 1,000 c.c. of milk.

DISCUSSION

The ascorbic acid content of milk produced by Guernsey and Holstein cows varying in age from four to 11 years does not

TABLE 4

Ascorbic Acid Content of Milk[1] as Influenced by Age of Cow—Holstein

Age		Aug. 19	Sept. 16	Sept. 30	Oct. 14	Oct. 28	Nov. 10	Nov. 24	Dec. 9	Dec. 23	Jan. 6	Jan. 20	Feb. 3	Feb. 17	March 3	March 23	April 7	April 21	May 6	May 19	June 2	June 23	July 8	July 21	Aug. 11	Group av.
yr.																										
5	No. of cows.....	.....	.....	.....	.....	.....	1	2	2	2	2	1	1	1	1	1	1	3	3	3	2	1	1	1	1	15.30
	Min.....	.....	.....	.....	.....	.....	.....	16.00	17.60	14.00	14.30	.....	.....	.....	.....	.....	.....	9.84	11.52	16.02	10.70	.....	.....	.....	.....	30 assays
	Max.....	.....	.....	.....	.....	.....	.....	16.30	17.91	15.30	15.30	.....	.....	.....	.....	.....	.....	16.10	17.50	19.40	15.90	.....	.....	.....	.....	4 cows
	Av.....	.....	.....	.....	.....	.....	16.40	16.12	17.74	14.63	14.80	16.41	16.51	15.80	14.11	13.41	11.90	13.70	15.01	18.02	13.30	11.00	15.90	17.30	17.12	
6	No. of cows.....	6	7	7	6	6	5	4	4	4	4	4	4	4	4	4	6	6	6	6	6	6	6	6	4	18.62
	Min.....	15.44	15.80	15.02	14.51	17.90	16.40	18.60	15.30	14.31	14.00	13.50	14.10	15.80	13.72	11.50	13.74	13.12	15.40	16.02	15.53	15.43	16.90	14.62	17.12	125 assays
	Max.....	21.32	20.12	20.02	22.00	23.03	23.40	21.17	23.44	19.74	20.80	22.62	23.54	24.91	21.00	19.60	19.70	19.40	18.20	21.40	19.33	19.90	21.60	19.50	21.10	10 cows
	Av.....	17.70	17.73	18.23	19.64	20.30	20.60	20.40	20.92	19.74	18.60	19.61	20.60	20.70	18.60	16.60	17.80	16.51	16.70	18.91	18.01	17.71	18.50	17.40	19.21	
7	No. of cows.....	.....	1	1	1	1	2	1	2	2	1	1	1	2	2	2	1	1	2	2	2	2	2	2	2	18.91
	Min.....	.....	.....	.....	.....	.....	20.10	.....	15.63	15.61	.....	.....	.....	22.84	19.44	18.40	.....	.....	16.10	19.03	16.91	15.10	17.20	17.72	18.00	36 assays
	Max.....	.....	.....	.....	.....	.....	20.60	.....	20.20	17.90	.....	.....	.....	23.20	19.82	18.40	.....	.....	16.80	19.40	18.64	18.51	19.24	18.20	20.63	3 cows
	Av.....	.....	19.04	19.30	20.60	20.10	20.38	19.22	17.91	16.80	20.14	23.00	21.10	23.01	19.63	18.40	18.60	16.40	16.41	19.20	17.80	16.80	18.22	17.94	19.32	
8	No. of cows.....	2	2	2	2	2	2	3	2	2	2	3	3	3	3	3	3	2	2	2	2	2	2	2	2	19.10
	Min.....	18.40	20.70	21.20	19.61	22.30	21.50	19.60	21.82	16.90	19.50	18.24	19.00	19.51	16.80	16.90	14.90	16.40	15.02	16.02	16.60	16.50	18.20	15.10	14.10	55 assays
	Max.....	20.60	21.60	22.14	23.13	22.30	24.80	21.82	21.82	18.20	20.14	21.52	20.40	22.42	20.20	19.94	17.10	16.73	15.71	18.03	18.00	18.20	18.20	16.61	17.12	4 cows
	Av.....	19.50	21.10	21.70	21.40	22.30	23.13	20.85	21.82	17.60	19.82	20.20	19.80	21.50	18.43	18.70	16.10	16.60	15.40	17.03	17.30	17.31	18.20	15.84	15.60	
9	No. of cows.....	3	4	4	3	4	4	4	4	4	5	5	5	5	5	5	3	2	2	3	3	4	4	3	3	17.60
	Min.....	14.34	15.50	16.60	15.70	16.73	16.82	18.30	18.23	12.40	16.90	16.10	16.90	16.61	15.64	13.80	16.34	14.43	15.40	11.70	14.84	14.10	14.84	16.00	16.24	91 assays
	Max.....	17.30	19.40	22.33	18.63	25.30	20.10	21.20	21.20	18.90	20.50	20.80	20.73	20.80	20.10	18.01	18.93	16.10	16.41	18.03	18.00	18.20	18.22	16.84	17.60	6 cows
	Av.....	16.00	16.84	18.80	17.50	19.90	18.60	19.31	19.40	15.93	18.33	19.30	18.83	18.93	18.00	16.20	17.70	15.30	15.90	15.02	16.22	15.70	16.50	16.30	17.00	

[1] Milligrams per 1,000 c.c. of milk.

show any consistent relationship between the age of a cow and ascorbic acid content of the milk that she produced. This conclusion is substantiated by Whitnah and Riddell (1937) who state that the relationship of vitamin C content of milk to the age of the cow "is small and unimportant." Rasmussen and associates (1936) also found no consistent relationship between the age of the cows of five different breeds and the ascorbic acid content of their milk. However, it is interesting to note in this connection that the average ascorbic acid content of the milk reported by Rasmussen for the college herd was relatively low for all the breeds, and the values obtained for the Guernsey and Holstein cows were only about one-half the yearly values obtained for these breeds in this investigation.

SUMMARY

A study was made of the ascorbic acid content of milk produced by mature Guernsey and Holstein cows of different ages.

The experimental cows were maintained, as a part of a large certified milk herd, for a 12-months period.

The cows were stall-fed, a uniform ration consisting of a variety of hays and concentrates. They had no access to pasture and were out of the stables only for exercise.

The cows and attendants were under continuous veterinary and medical inspection. Particular attention was given to the condition of the cows' udders.

The age range of the cows was four to 11 years, inclusive, for the Guernseys and five to nine years for the Holsteins.

The results of the assays of 659 samples of milk for their ascorbic acid content did not reveal any consistent relationship between the age of a cow and the amount of ascorbic acid in her milk.

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DENSITY OF DRY MILK SOLIDS[1]

OLOF E. STAMBERG[2]
AND
C. H. BAILEY
Division of Agricultural Biochemistry, University of Minnesota, St. Paul, Minnesota

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In an attempt to study the particle-size distribution of various types of dry milk solids[3] by the sedimentation method, it was found that the method could not be used owing to the variation in density of the individual particles of the samples. These observations were made on samples of dry milk solids made by the spray process, and led to further investigations on the density of the particles.

Griebel (1916) noticed that some dry milks contained small air cells. Palmer and Dahle (1922) observed through the microscope that dry whole milk made by the spray process contained air cells and that no air cells were present in the irregular particles of dry milk made by the drum process. Fogler and Kleinschmidt (1938), in an excellent article on spray drying, state that the particles formed by various commercial spray processes are either hollow or porous in the interior to a varying degree and that the density of the products vary. Thus it becomes evident that the particles of spray-process dry milk solids vary in apparent density owing to occluded air cells.

Jack (1939) studied the volume contraction of spray-process and roller-process dry milk solids in water by using a dilatometer, and concluded that the volume contractions of 27 to 36 cubic millimeters per gram of the roller milk solids and 51 to 82 cubic millimeters per gram of spray milk solids were due mainly to the absorption of water by the milk proteins. The author does not mention that the occluded air in the milk solids was corrected for, and since the material stood for 20 to 22 hours in the dilatometer, it is quite possible that the rise of air bubbles was to a certain extent a factor in the lowering of the volume in the capillary. This appears more probable since the spray-process samples gave much higher values with an appreciable variation.

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[1] Paper No. 1739, Journal Series, Minnesota Agricultural Experiment Station, St. Paul.

[2] American Dry Milk Institute Fellow, University of Minnesota.

[3] Dry milk solids, not over one and one-half per cent fat, is the product resulting from the removal of fat and water from milk. It contains not over one and one-half per cent butterfat and not over five per cent moisture.

EXPERIMENTAL PROCEDURE

Four samples of dry milk solids were prepared by the regular plant operators in a commercial plant, using the Merrell-Soule spray process, from the same supply of milk. Different heat treatments were used in preparing these samples. Sample P-150 was preheated to 65.6°C.(150°F.) and Sample P-200 to 93.3°C.(200°F.). Sample S-150 was first preheated to 150°F. and then superheated by a steam treatment, while Sample S-200 was preheated to 200°F. and then superheated. The holding time during the preheating was constant for all samples, but it was not definitely recorded. In addition to the samples of known heat treatments a high-quality commercial sample of spray-process dry milk solids was used as a standard, and a commercial roller-process product was also used.

A series of mixtures of naphtha and carbon tetrachloride was prepared with densities of 1.1, 1.2, 1.3, 1.4, and 1.5, respectively, at 25°C.(77°F.). Three grams of dry milk solids and 20 c.c. of the density medium were placed in small test tubes, which were stoppered and shaken thoroughly. After standing 30 minutes the particles separated into a layer lighter than the medium and a precipitate heavier than the medium. These tests were made with all the milk samples at the five densities. The distribution of the particles varied with the samples. Sample P-150 had less of the heavier particles, with Samples P-200, S-150, S-200, and the standard sample having increasingly more of the heavier particles in the order named. In all of these samples some particles had densities of less than 1.1 and higher than 1.4. but all particles were of a lower density than 1.5. In the case of the roller product all particles were precipitated in the medium with a density of 1.4 and floated on the medium with a density of 1.5, thus showing no great variability in the density of the particles.

One sample each of spray-dried and roller-dried milk solids was thoroughly pulverized by grinding in a rod mill for 24 hours. Employing a pycnometer, with carbon tetrachloride as the fluid medium, the actual average densities of these ground, air-free products were 1.470 when made from roller-dried products and 1.459 when made from spray-dried products, respectively. This indicates that the previously observed density distribution of the original unground particles in the spray milks was largely influenced by occluded air. This was further verified by observing the spray particles under the microscope while a drop of water was placed on the slide. Air cells could not be noticed in the dry particles, but as the particles dissolved the air bubbles appeared. The rather large size of many of the air bubbles indicated that they were probably present in the

particles under a pressure higher than atmospheric pressure. The number of occluded air cells and the samples was related to the results of the density tests, in that Sample P-150 had the most air cells and the standard sample the least. The roller product had only a very few isolated air cells.

A method was then developed whereby the density distribution of the particles of spray-process dry milk solids could be expressed as a relative numerical value.

It was found that a mixture of naphtha and carbon tetrachloride with a density of 1.250 at 25°C.(77°F.) gave the best differentiation in the amount of precipitate with various samples. The 50-c.c. graduated conical sedimentation tubes now used for the solubility index

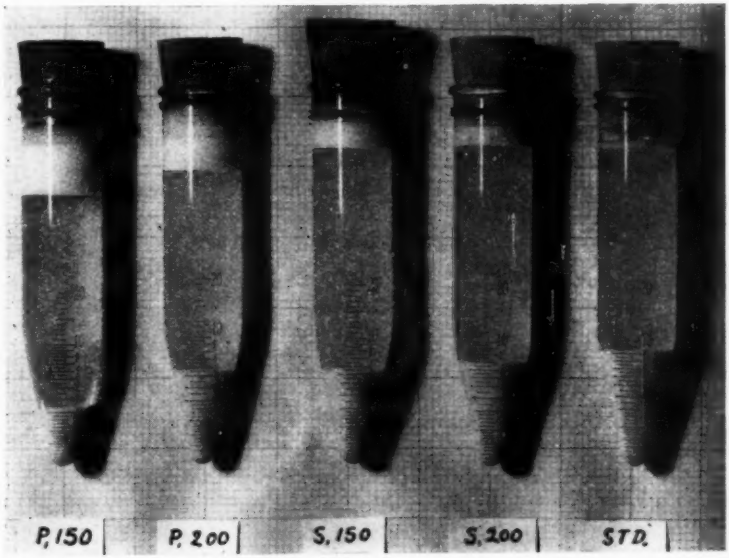

FIG. 1. Tubes used in determining the density index, and results obtained with the five spray powders in a liquid medium with a density of 1.250 at 25°C. (77°F.).

of dry milk solids—American Dry Milk Institute, Inc. (1936)—were employed, seven grams of dry milk solids (not over five per cent moisture) were placed in each, and the density mixture was added to the 50-c.c. mark. The tubes were stoppered, shaken thoroughly, allowed to stand in a level position for 45 minutes, and the precipitates were then read to the nearest tenth of a cubic centimeter. The value thus obtained times 10 was called the "density-index" of the dry milk solids; that is, c.c. precipitate $\times$ 10 = density index.

This method of expressing the values as 10 times the volume in cubic centimeters of the precipitate approximates the percentage of the milk solids with an apparent density higher than 1.250. It was not found advantageous to centrifuge the tubes, as the values thus

TABLE 1

Density Indices, Percentage of Particles of Density Higher Than 1.250, and Manufacturing Process of 30 Samples of Spray-Process Dry Milk Solids

No.	Density index [1] X	Precipitate Y	Manufacturing process
		pct.	
1	125	84	G [2]
2	118	80	G
3	118	89	M
4	118	90	G
5	105	86	G
6	101	91	R?
7	97	82	G
8	96	92	M
9	94	80	G
10	84	84	G
11	83	83	M
12	81	80	G
13	80	83	R
14	76	72	....
15	76	71	R
16	72	50	M
17	71	66	G
18	70	66	R
19	68	46	R?
20	68	69	G
21	68	64	M
22	62	75	R
23	59	53	....
24	55	54	R?
25	55	49	M
26	54	49	G
27	50	48	R
28	44	41	....
29	34	36	R
30	24	20	....

[1] $r_{xy} = .88 \pm .028$. [2] G = Grey-Jensen, M = Merrell-Soule, R = Rogers.

obtained were relatively the same. The density medium can be recovered by filtration and used again after the density has been checked and adjusted.

In the tubes with the five samples of spray-process product in a medium having a density of 1.250 at 25°C. (Fig. 1) the density in-

dices of P-150, P-200, S-150, S-200, and standard were 24, 44, 55, 58, and 83, respectively.

This method was employed to determine the variation in the density index of numerous commercial spray products from different manufacturers, employing 30 samples for this purpose. The density indices were determined and also the actual percentages of the heavier precipitated fractions, which were quantitatively recovered and dried to constant weight at 80°C.(176°F.). The results (Table 1) show that the coefficient of correlation (r) between the density index and the percentage of dry milk solids of higher apparent density than 1.250 was $r = .88 \pm .028$, thus showing a high correlation. The fourth column (Table 1) records the manufacturing method used as being Gray-Jensen, Rogers, or Merrell-Soule. Thirty-one additional spray samples from different manufacturers showed a similar variation in density indices ranging from 28 to 112.

DISCUSSION

It is quite evident that the particles in dry milk solids, not over one and one-half per cent fat, made by the spray process vary in apparent density owing to occluded air cells, but practically no air is present in the particles made by the roller process. Thus the manufacturing method appears to be the factor causing this difference. The density index was higher with a greater degree of preheat treatment with the four samples made from the same milk supply, by the same equipment, and prepared with a variation only in the treatment. Our observations, while restricted to a few samples, indicate that the density index can be used as a quick and easy test for uniformity of the products. The manufacturer is in the best position to study this possibility further. The data (Table 1) show that there is a rather wide variation in density index of commercial samples and that this factor varies with all of the three spray processes used.

SUMMARY

Particles of dry milk solids, not over one and one-half per cent fat, made by the spray process vary in apparent density owing to occluded air cells, but practically no air cells were observed in particles by the roller process.

A method was developed for the determination of the density index, which is a relative value for the distribution of particles of higher and lower apparent density than 1.250 and approximates the percentage by weight of particles of higher density than 1.250.

Commercial dry milk solids made by the spray process were found to vary widely in the density index.

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PALATABILITY AND COLOR OF POTATOES BOUGHT ON A RETAIL MARKET

RUTH M. GRISWOLD
Section of Home Economics, Department of Foods and Nutrition, Michigan Agricultural Experiment Station, East Lansing, Michigan

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The large consumption of potatoes in this country makes it important to know what kind of potatoes the consumer prefers and what qualities make certain potatoes desirable to him. A number of papers have appeared in the literature but additional work seems desirable. The measurement of palatability is still largely subjective, depending on scoring by judges for quantitative data. Score cards, varying in complexity, have been used, and the Potato Association of America is attempting to work out a standard score card in coöperation with investigators engaged in potato research. An interesting departure from the usual grading charts has been made by Sweetman (1936) who uses a subjective test for mealiness only, omitting the other palatability factors. The importance of this factor in determining the quality of potatoes cannot be denied, considering the evidence given by Sweetman and others of the interrelationships between mealiness and other palatability factors.

Some objective tests of the quality of cooked potatoes have been developed to supplement or replace scoring by judges. Rathsack (1935) measured the tenderness of cooked potatoes by determining the length of time required for the penetration of a weighted, stretched wire through a cube of the tuber, while Barmore (1937) and other workers have used a penetrometer. Several methods have been employed for measuring the color of potatoes. McKay (1931) used the Munsell system, while Bilham, Maunsell, and Lampitt (1937) developed an ingenious method using a Zeiss-Pulfrich photometer.

This study was undertaken to determine whether a relationship exists among palatability factors graded by judges and between these factors and color measured objectively in potatoes grown in three states. Such an analysis may simplify potato testing by showing that it is possible to omit certain factors very well correlated with each other from the scoring, and it may also clarify ideas on what qualities make a potato palatable.

EXPERIMENTAL PROCEDURE

The potatoes used in this study were bought on the retail markets of Detroit between the middle of February and the middle of May,

1937. Fifty 10-pound or one-peck samples of "old" potatoes grown in Michigan, Maine, and Idaho, designated as A, B, and C, respectively, were purchased and tested, making a total of 150 samples.

The inedible portion was determined by cutting away bruised or decayed portions, but the peel and small scratches were not removed. The boiling, baking, and sampling methods were essentially those used by Wright, Peacock, Whiteman, and Whiteman (1936). Five potatoes of fairly uniform size from each sample were boiled without peeling in an enamel pan containing one and one-half quarts of distilled water. Boiling was continued until a thermometer inserted through the lid into one potato registered 96°C.(204.8°F.). Five more potatoes from each sample were baked in a gas oven at 204.4°C.(400°F.) until a thermometer inserted in one potato registered 100°C.(212°F.). The tubers were cut in half lengthwise immediately after cooking. One set of halves was left intact and shown to the judges, while the other half was riced for testing. No salt was added at any time. The potatoes were identified by numbers in such a way that the judges could not know which boiled and baked potatoes came from the same lot.

The grading charts were worked out with the coöperation of the judges and are similar to those used for meat judging in the National Coöperative Meat Project. Palatability is divided into several factors which are graded on the basis of their intensity and desirability wherever possible. Wright, Peacock, Whiteman, and Whiteman (1936) furnished the score card used for their bulletin, and several features from this card were used. The grading chart for boiled potatoes is shown (Fig. 1); the chart for baked potatoes is the same except that sloughing is replaced by tenderness of skin, which is graded from very tough (1) to very tender (5). The judges felt that no less than five gradations of each point would serve their purpose and, after using the score for some time, wanted still finer gradations. They were permitted to indicate a score half way between two grades whenever they wished.

The same four or five judges, with one exception, did the scoring throughout the experiment. All of them were college staff members, and several had served on other palatability committees.

The color of the mixed, riced potato was judged, when cold, in a Lovibond tintometer. In this instrument, light is reflected from the surface of the sample and from the surface of a standard white substance, magnesium carbonate. The light reflected from the standard passes through colored slides which are adjusted to match the sample. Yellow, red, blue, and neutral slides of various intensity are

FIG. 1. Grading Chart for Boiled Potatoes

Cooking Laboratory No.....Sample No.....Date.....

Check one item in each group:

Quality		5	4	3	2	1
Sloughing	Skin	Intact	Slightly broken	Moderately broken	Broken with slight sloughing	Much breaking and sloughing
Color	Intensity	Very pronounced	Pronounced	Slightly pronounced	Perceptible	Imperceptible
	Desirability	Very desirable	Desirable	Slightly desirable	Slightly undesirable	Undesirable
Mealiness	.....	Very good	Good	Fair	Poor	Very poor
Texture	Intensity	Very pronounced	Pronounced	Slightly pronounced	Perceptible	Imperceptible
	Desirability	Very desirable	Desirable	Slightly desirable	Slightly undesirable	Undesirable
Flavor of mashed flesh	Intensity	Very pronounced	Pronounced	Slightly pronounced	Perceptible	Imperceptible
	Desirability	Very desirable	Desirable	Slightly desirable	Slightly undesirable	Undesirable
General conclusion	.....	Very good	Good	Fair	Poor	Very poor

Check one or more items in each group:

Color
- White
- Cream
- Yellow
- Pink
- Slightly grey
- Grey
- Dark grey

Location of blackening
- Cortex
- Medulla
- Stem end
- Bud end
- Whole potato

Texture
- Mealy
- Waxy
- Soggy
- Hard
- Tough

Remarks.....

.....

.....

.....

Signature.....

furnished with the instrument. Each slide is numbered, the highest numbers representing the deepest colors.

RESULTS AND DISCUSSION

The palatability scores and objective data for potatoes from the three states and the combined 150 samples are summarized (Table 1). The palatability scores, being subjective, are likely to contain rather large discrepancies because judges have personal standards for the

TABLE 1

Palatability Scores and Objective Data for Potatoes from Michigan, Maine, and Idaho

	State A		State B		State C		Average	
Price per peck	0.44		0.52		0.66		0.54	
Percentage inedible	4.83		3.18		3.23		3.75	
Method of cooking	Boiled	Baked	Boiled	Baked	Boiled	Baked	Boiled	Baked
Judges' scores								
Sloughing of skin	4.4	....	4.0	....	4.6	....	4.3	....
Tenderness of skin	....	3.0	....	2.8	....	3.1	....	3.0
Color								
Intensity	3.5	3.3	3.4	3.1	3.0	3.1	3.3	3.2
Desirability	2.1	2.5	2.3	2.9	3.2	3.7	2.5	3.0
Mealiness	2.6	3.0	2.8	3.0	3.8	3.8	3.1	3.3
Texture								
Intensity	3.0	3.0	2.9	3.0	3.4	3.5	3.1	3.2
Desirability	2.7	2.9	2.8	2.9	3.7	3.7	3.1	3.2
Flavor mashed flesh								
Intensity	3.1	3.0	3.0	3.1	3.1	3.2	3.0	3.1
Desirability	2.4	2.6	2.6	2.6	3.4	3.3	2.8	2.8
General conclusion	2.3	2.6	2.5	2.7	3.5	3.5	2.8	2.9
Tintometer readings								
Yellow	1.52	1.54	1.18	1.23	1.29	1.22	1.33	1.33
Blue	0.02	0.00	0.02	0.00	0.00	0.00	0.01	0.00
Red	0.30	0.36	0.20	0.27	0.21	0.21	0.24	0.28

product being tested that may not be the same as those of other judges and because a judge frequently is not consistent in replicate determinations on the same product. These errors can be examined by analysis of variance,[1] which is a method of separating variations owing to different factors and obtaining a good estimate of experimental error because variations owing to known causes are removed. The means are tested for significance, using this estimate of error. Analyses for various palatability factors show that the variation

[1] Fisher's method (1936) for analysis of variance and Snedecor's F (1937) were used.

between judges is highly significant, indicating that judges were following different standards but each judge was reasonably consistent in his or her own work.

Since many palatability factors were graded, it seemed desirable to find how these factors were related to each other. This was done by a statistical method of finding correlations, which measure the relationship between two or more variables. A perfect correlation is plus or minus one, while a correlation of zero shows that there is no relationship between the factors. A positive correlation shows that the variables increase together, and a negative one shows that as one factor increases the other decreases.

Correlations between the scores for various items of palatability are of theoretical interest in determining what factors make up taste

TABLE 2

Correlations of Several Palatability Factors

Correlation between	Boiled potatoes	Baked potatoes
Mealiness and sloughing of skin	—.123	—.119
Mealiness and desirability of color	.766 [1]	.760 [1]
Mealiness and desirability of texture	.927 [1]	.923 [1]
Mealiness and desirability of flavor	.789 [1]	.771 [1]
Mealiness and "general conclusion"	.859 [1]	.846 [1]
Intensity and desirability of color	—.596 [1]	—.174 [2]
Intensity and desirability of texture	.555 [1]	.577 [1]
Intensity and desirability of flavor	—.049	—.157
Price per peck and "general conclusion"	.693 [1]	.583 [1]
Percentage inedible and "general conclusion"	—.108	—.250 [1]

[1] Highly significant. The significance of these correlations has been determined according to Fisher (1936). [2] Significant.

preferences as well as of practical importance in developing testing methods. The correlations between mealiness and the other palatability scores are of special interest, considering the emphasis placed on mealiness by the American consumer and by many investigators. Several investigators have found that very mealy potatoes slough badly. Sweetman (1936) states that sloughing often parallels mealiness in amount and that low cohesion tends to be associated with mealiness. She also reports that Dow obtained a correlation of .469 $\pm$ .11 between sloughing of potatoes boiled without skins and mealiness of baked tubers in the same lot. However, correlations in the present study between sloughing of the skin and mealiness of baked potatoes are not significant (Table 2). This may be explained by the experimental method used which minimizes sloughing by boiling potatoes in their skins and by cooking them until just done as

shown by the internal temperature. The long storage to which the potatoes had been subjected, since they were purchased during the late winter and early spring, also tended to decrease sloughing. Rathsack's work (1935), recently confirmed by Barmore (1938), indicates that the degree of sloughing decreases very rapidly with increasing storage time because the shrinkage taking place during storage makes possible expansion in cooking without disintegration.

The results show that when scores for mealiness are high, grades for desirability of color, texture, flavor, and "general conclusion" are also high. Since the correlations of mealiness with desirability of texture are so high, it may be concluded that judges considered these terms synonymous for the potatoes tested. This would probably not be true of new potatoes, in which waxy rather than mealy texture may be desirable.

Several investigators—Child and Willaman (1929), Patton and McKay (1934), Cobb (1935), and Sweetman (1936)—reported high correlations between mealiness or texture and desirable flavor. Sweetman (1936) stated that mealiness frequently is highly correlated with desirable color, but Child and Willaman did not find a significant correlation between color and texture.

The correlations between intensity and desirability of color are negative for both boiled and baked potatoes, indicating that the judges considered a light color desirable. Intensity and desirability of texture show highly significant positive correlations. The correlations of intensity with desirability of flavor are not significant. Marked differences between the correlations for the three states are noticed in this case. For boiled potatoes the correlations are —.300 for State B, —.529 for State A, and .321 for State C. The corresponding correlations for baked potatoes are —.507, —.503, and .253. For both boiled and baked potatoes the correlations of intensity with desirability of flavor for State C are significantly different from those of States A or B. This indicates that the potatoes from State C are quite different from those from States A or B in the relationship between intensity and desirability of flavor. A possible explanation is offered (Table 1), which shows that the judges considered the flavor of State C potatoes better than that of potatoes from States A or B. Evidently with the State C potatoes an intense flavor is desirable, and with the potatoes from States A or B an intense flavor is less desirable.

As might be expected, the more expensive potatoes have higher palatability scores than cheaper potatoes, as shown by highly significant correlations between "general conclusion" and price. One might also expect potatoes with a large amount of waste to be less

palatable. Apparently this is true for baked potatoes and not for boiled, as the correlation between percentage inedible and "general conclusion" is significant and negative for the former and not significant for the latter. There is not a significant relationship between price per peck and per cent inedible, the correlation being —.120.

Child and Willaman (1929) report highly significant correlations between the scores of baked and boiled potatoes for color, flavor, texture, and total score. The data obtained in the present study show highly significant correlations between boiled and baked potatoes of .752 for mealiness and .777 for "general conclusion," indicating that if a potato is of good quality when boiled, it is likely to be of good quality when baked. This finding is not in complete agreement with the common belief that potatoes which bake well do not boil well, usually owing to excessive disintegration. As already mentioned, sloughing was less in this experiment than would be expected under most conditions. New potatoes which are good when boiled probably would not be good when baked, owing to their special texture.

The quality of the potatoes, as measured by the "general conclusion" for boiled potatoes, declined slowly during February and March and more rapidly during April and May. An analysis of variance showed highly significant differences between months as well as between states. The potatoes were significantly less palatable in May than in February, March, or April but were not significantly different in February, March, and April. Similarly McKay and Patton (1936) found that scores were lower for all potato samples during the spring regardless of time of planting or variety.

The objective color data (Table 1) show that in addition to yellow and red, blue slides were sometimes needed to match the color of the sample in the Lovibond tintometer. When blue was necessary, the sample was quite grey. In the instrument used, light reflected from the standard was slightly greyed by passing through the slides, causing a slight greyness to escape exact measurement. It did not seem possible to take the figures for blue into consideration in the correlations, since they occur in only a few samples, so all data on samples for which blue was used have been omitted. No blue was needed to match the baked potatoes from State A or the boiled or baked potatoes from State C. Seven boiled samples from State A were matched using blue, nine boiled samples from State B, and one baked sample from State B. The Munsell system or the photometer used by Bilham, Maunsell, and Lampitt (1937) would undoubtedly measure the greyness of potatoes more accurately than the Lovibond tintometer.

The figures obtained with this instrument proved valuable in spite of their limitations. Statistical tests of the significance of differences between the color of potatoes from the three states were complicated by the fact that both yellow and red were used and that it was not possible to average these figures. Therefore it was necessary to test for significance between pairs of numbers instead of between single figures. A new application of Rietz's (1927) method of ellipses was made by Dr. W. D. Baten of Michigan State College. Ninety-nine per cent of the cases were included instead of 50 per cent. It was found that State A potatoes, both boiled and baked, were considerably darker in color than potatoes from the other two states. Baked potatoes from State B were significantly darker than baked potatoes from State C. It was questionable whether the colors

TABLE 3

Multiple Correlations Between Objective Color Data and Several Palatability Factors

Multiple correlation between yellow-red and judges' scores for	Boiled potatoes	Baked potatoes
Intensity of color	.408 [1]	.281 [1]
Desirability of color	.341 [1]	.570 [1]
Mealiness	.438 [1]	.516 [1]
Desirability of flavor	.375 [1]	.457 [1]
General conclusion	.394 [1]	.518 [1]

[1] Highly significant. The significance of these correlations has been tested according to Snedecor (1937).

of boiled potatoes from States B and C were significantly different.

Correlations between yellow and red are high, being .787 for boiled potatoes and .809 for baked potatoes. It is evident from these correlations that if a potato contains a large amount of yellow, it is likely to contain a large amount of red also.

Since the objective color data consist of both yellow and red, correlations between color and palatability data are multiple (Table 3). Highly significant relationships are found in every case between palatability factor and color. A light color measured on the tintometer was associated with low scores for intensity of color and high scores for the other palatability factors, showing that potatoes which the judges graded low in color intensity actually proved to have a light color when measured in the tintometer and that light color is associated with desirable color, good mealiness, desirable flavor, and a favorable "general conclusion." In other words, a potato which has little color is likely to receive a good grade for palatability from a group of judges. This preference for very white potatoes is more

marked in America than in some other countries, such as Germany, where Rathlef (1934) says yellowish potatoes are preferred. The conclusions regarding the multiple correlations are based on the signs of the regression coefficients in the predicting equation. These signs must be found since it is mathematically impossible to give a plus or minus sign to a multiple correlation. Both regression coefficients are positive for the correlation of yellow-red and judges' scores for intensity of color with boiled and baked potatoes. Both coefficients are negative for every other correlation (Table 3).

SUMMARY

One hundred and fifty samples of potatoes bought on the Detroit retail market were boiled, baked, scored by judges, and tested objectively for color. The data were analyzed statistically.

Mealiness shows highly significant positive correlations with other desirable palatability factors, indicating that accurate determinations of mealiness made on a large sample would be confirmed by a group of judges scoring the potato on various palatability factors.

Palatability increased with price in the samples tested.

The correlations between boiled and baked potato scores are highly significant both for mealiness and general conclusion, indicating that if a potato of the type used in this experiment is of good quality when boiled it is also likely to be of good quality when baked.

Palatability declined with storage from February to May.

The color data correlate significantly with judges' scores for palatability, showing that a comparatively white potato will probably be scored higher by a group of judges than a darker potato.

ACKNOWLEDGMENT

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PHYSICOCHEMICAL CHANGES IN UNFERTILIZED, INCUBATED EGGS OF *GALLUS DOMESTICUS*

ALEXIS L. ROMANOFF
Department of Poultry Husbandry, Cornell University, Ithaca, New York

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On an average about 12 per cent of all chicken eggs set for hatching are sterile. These otherwise "good" eggs, when removed after a few days of incubation, are usually discriminated against on the market. In commercial hatching there are annually incubated over 100,000,000 infertile eggs. These eggs, therefore, are a by-product which can be disposed of for industrial and other purposes only at a much lower price.

The present study was undertaken to determine the rate of chemical decomposition of incubated or preheated, unfertilized eggs and to compare their quality and value with cold-storage eggs.

MATERIALS AND METHODS

About 1,260 unfertilized eggs of White Leghorn hens *(Gallus domesticus)* were used. The eggs were selected for the average weight of 60 ± 2 grams, with normal shell texture, and were not over 24 hours old.

The study consisted of two parts: (1) the loss in weight of whole eggs, including the time and rate of loss in weight to complete dehydration, and the relative loss in weight under various conditions; and (2) the progressive changes in physicochemical properties of egg content during 21 days of incubation.

In the first part of the study the selected eggs, in groups of 12, were dried at 45 per cent relative humidity and at temperatures of 20, 37.5, and 55°C.(68, 99.5, and 131°F.). At weekly intervals the eggs were weighed individually on torsion balance up to .01 gram. Also the eggs, in groups of 30, selected for size and shell permeability, were incubated 21 days at different humidity, temperature, and rate of air movement. These eggs were daily weighed collectively on sartorius balance up to .01 gram.

In the second part of the study about 960 eggs, in groups of 160, were incubated in the electric laboratory incubator, Romanoff (1929), at the following conditions: temperature 38 ± .2°C., relative humidity 60 ± 1 per cent, low content of carbon dioxide and velocity of air around the eggs not exceeding .1 mile per hour. At intervals of 24

hours from 30 to 50 eggs were used for a series of analyses by the following most suitable methods: (1) proportional amounts of yolk and albumen were determined by dissection of a coagulated egg after 20 minutes of boiling; (2) the elasticity of vitelline membrane was determined by laying the yolk on a plane surface and measuring its height by a spherometer and its width by a micrometer caliper; (3) the moisture and dry-matter content were obtained by the drying of about two grams of egg substance to constant weight in a Fries vacuum oven at 55°C. and vacuum 62.5 cm.; (4) specific gravity of the egg contents were determined by weighing samples in a small five-c.c. pyknometer at 25°C.(77°F.); (5) the refractive index was read at 25°C. with a Zeiss refractometer, Abbe type; (6) the hydrogen-ion concentration was determined electrometrically, using a hydrogen electrode; (7) the ammonia content of yolk and albumen was determined by the aerometric method of Folin, using Nessler solution as the reagent; (8) the calcium, as calcium oxide (CaO), in yolk and albumen was estimated gravimetrically from the total ash by the modified McCrudden method, Romanoff (1930); (9) the total nitrogen was determined by the Kjeldahl method; (10) the fat was extracted with ether from dry samples of material by a Soxhlet extraction apparatus; (11) the extracted fat was tested for saponification number, Koettstorfer (1879), iodine value, Wijs (1898), and refractive index, by a Zeiss butter refractometer.

LOSS IN WEIGHT OF EGGS

The loss in weight of unfertilized eggs with age is due chiefly to loss of free water by evaporation. Besides loss of water there is a continuous elimination of gases, such as carbon dioxide—Atwood and Weakley (1924), Bohr and Hasselbalch (1900), and Smith (1930); ammonia—Bandemer and Schaible (1936), Jenkins and Pennington (1919), and Lythgoe (1927); and perhaps nitrogen—Hasselbalch (1900)—and hydrogen sulphide—Rubner, *et al.* (1893); absorption of oxygen—Hasselbalch (1900); and continuous elimination of heat —Langworthy and Barott (1921), as the result of partial organic decomposition.

Complete Dehydration: Eggs lose weight continuously with aging, from the time of being laid until their complete dehydration. The rate of loss in weight is influenced by environmental conditions among which temperature is of considerable importance. Observations showed that under a constant relative humidity of about 45 per cent the daily loss in weight at the beginning was .092 gram per egg at 20°C.(68°F.), .590 gram at 37.5°C.(99.5°F.), and .842 gram at 55°C.(131°F.). The average daily losses per egg during

the first month of aging under these temperatures were .098, .440, and .651 gram, respectively. It is evident that the daily losses diminished with the age of the egg (Fig. 1); also with the successive months of aging the decrease in the rate of daily loss in weight was more rapid at high than at low temperature.

On the whole, it took about 10 months to dry out the egg at 20°C., and a little over four months at 37.5°C. At 55°C. the complete dryness of the egg was reached only after 15 months. At this high temperature it soon became apparent that fats in the form of oil

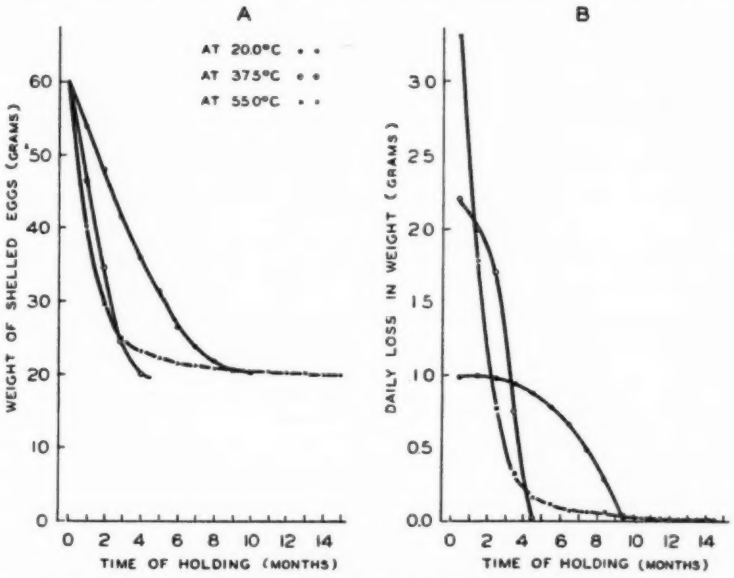

FIG. 1. Loss in weight of eggs from the time of laying to a complete dehydration at 45 per cent relative humidity and at temperatures of 20, 37.5 and 55°C. (68, 99.5, and 131°F.). A shows the average weight of eggs each month of holding; and B, the average daily loss in weight per egg during successive months of holding.

ooze through the shell, partially seal the pores, and in such way retard the dehydration of egg contents.

At the end of the drying periods the proportional weights of eggs were slightly higher at low than at high temperature (Table 1). These results indicate that the rapid rate of drying by a higher temperature gives greater loss of dry matter in the eggs through increased organic decomposition.

The data on the total loss in weight of different sizes of eggs dehydrated at 37.5°C. (Table 1) show that large eggs had actually

more but proportionally less dry matter, including shell, than small eggs. This was presumably due to the presence of proportionally large amounts of albumen in large-sized eggs—Curtis (1911), Atwood and Weakley (1917), and Tso (1925). Since the percentage of dry matter of albumen is less than of yolk, there would be proportionally less total dry matter in large eggs than in small ones.

Influence of Various Factors: The loss in weight of eggs was found to be influenced by several physical factors. Among these the most important are humidity, temperature, air movement, permeability of eggshell, and size of the egg (Table 2). The loss of a chief constituent—water—may be attributed to the evaporation effect

TABLE 1

Influence of Temperature and Size of Egg on Proportional Amounts of Dry Matter [1]

Observations		Dry matter of eggs		
		Original wet weight	Final dry weight	Percentage of dried weight
		gm.	*gm.*	*pct.*
Temperature	20°C.(68°F.)	59.81	20.07	33.56
	37.5°C.(99.5°F.)	59.91	20.01	33.40
	55°C.(131°F.)	59.83	19.85	33.18
Size of eggs [2]	large	69.64	22.48	32.28
	medium	59.82	20.05	33.52
	small	50.25	17.09	34.01

[1] There were 12 eggs in each group. The eggs were thoroughly selected for uniformity of original weight within .5 gram and good shell texture. [2] The eggs were dried at 37.5°C. They had reached the state of complete dryness at the same time, that is, in about 10 months.

with changes of humidity, air movement, and permeability of eggshell; to the vapor pressure with changes of temperature; and to the difference in surface area with changes of size of the egg.

The effect of humidity was evidently the greatest of all factors studied. Rough calculations indicate that within the observed limits the change in daily loss in weight of .01 gram per egg may be brought about by the change of either .6 per cent relative humidity, or 1.9°C., or 2.6 feet per second air movement; or 3 c.c./1 cm.^{2}/1 min. permeability of shell to air, or six grams of egg weight.

In addition to the above factors influencing the loss in weight of eggs may be mentioned the influence of altitude. Aggazzotti (1913) observed that fertilized eggs lost more weight each day at a high altitude (2,900 metres) than at sea level. This loss may be attributed to the difference in the rate of evaporation.

It also may be noted that there is no appreciable difference in the loss of weight between fertile and infertile eggs until about the 14th day of incubation. From the 14th day fertile eggs tend to lose more weight than infertile ones, Romanoff (1930), largely because of increased embryonic metabolism and increased permeability of eggshell.

PHYSICOCHEMICAL CHANGES DURING INCUBATION

With the loss in weight of eggs there are noticeable changes in the egg contents—in their structure, physical properties, and chemical composition. To ascertain some of these changes in unfertilized eggs incubated for three weeks the following daily observations on albumen and yolk were made: (1) proportional amounts, (2) dry matter, (3) percentage of water, (4) density, (5) refractive index, (6) hydrogen-ion concentration, (7) ammonia nitrogen contact, (8) elasticity of vitelline membrane, and (9) chemical changes, especially in fat, total nitrogen, and minerals during the whole period of incubation.

TABLE 2

Influence of Various Physical Factors on Loss in Weight of Incubated, Unfertilized Hens' Eggs[1]

Influencing factors		Loss in weight of eggs		
		Daily loss	Difference	Total loss in 21 days
		gm.	*gm.*	*pct.*
Standard conditions (av. of 124 eggs)[2]		.446	.....	15.61
Relative humidity	80	.357	.226	12.50
	40	.583		20.41
Temperature	41.5°C.(106.7°F.)	.516	.172	18.06
	32.5°C.(90.5°F.)	.344		12.04
Air movement	10 feet per second	.496	.026	17.36
	.1 foot per second	.470		16.45
Permeability of shell to air	25 c.c./1 cm.²/1 min.	.509	.067	17.82
	5 c.c./1 cm.²/1 min.	.442		15.47
Size of egg	70 grams	.495	.120	14.85
	50 grams	.375		15.75

[1] In all groups the standard conditions of incubation were as follows: (1) relative humidity 60 per cent; (2) temperature 38°C.; (3) air movement .1 foot per second; (4) size of eggs 60 ± .5 grams; and (5) low permeability of eggshell. [2] There were 30 selected eggs in each group, except with the standard conditions where there were 124 eggs.

Proportional Amounts of Albumen and Yolk: During incubation at 38°C.(100.4°F.), 60 per cent relative humidity, and slow air

movement, there was daily loss in weight on an average of .446 gram per egg. This change in weight can be attributed only to the loss in weight of albumen and yolk. The weight of the shell of an unfertilized egg is practically unchangeable during incubation except through a small loss of carbon dioxide—Atwood and Weakley (1924).

There is a linear decrease in wet weight of total egg contents (Fig. 2-A). This decrease was largely due to rapid loss in weight of albumen, because the weight of yolk was increasing up to about the 10th day. At this time the yolk gained in weight over 13 per cent. Then the weight of yolk also went down gradually and fell below its original weight. During the entire period of incubation the

TABLE 3

Effect of Incubation on Elasticity of Vitelline Membrane[1]

Incubation time	Shape of egg yolk		
	Height	Width	Height / Width
days	*mm.*	*mm.*	*index*
0	14.5	39.0	0.37
1	12.3	42.3	0.29
2	11.5	46.0	0.25
3	9.4	53.0	0.18
4	9.0	56.5	0.16
5	8.3	58.5	0.14
6	6.5	71.0	0.09
7	6.0	73.0	0.08
8	Vitelline membrane ruptures		

[1] Measured by the height and width of the yolk laid on a plain surface. There were 10 eggs used daily.

proportional weight of yolk to the weight of albumen had changed from about 56 to 70 per cent (Fig. 2-B).

Dry Matter: The total dry matter of egg contents was decreasing with incubation, and the rate of decrease was almost lineal during entire period (Fig. 3-A). This change was largely due to escape of some products of organic decomposition. The dry matter of yolk was decreasing while that of albumen was continually increasing. The increase of dry matter of albumen can be explained by the diffusion from yolk through vitelline membrane into albumen of glucose, fatty acids, and partially denatured or broken-down proteins. Pucher (1928) found that during 20 days of incubation of unfertilized eggs the total glucose of the albumen fell by 90 mgm. per cent and the total glucose of yolk rose by 40 mgm. per cent. Similar changes were found in the amounts of fat and total nitrogen (Table 4).

Water Content: The percentage of water in albumen was decreasing, while that of yolk was increasing with incubation (Fig. 3-B). The loss of water in the albumen may be easily accounted for by evaporation through the shell. From the fact that there is a pronounced increase of water in yolk, it is evident that there is a transfer of water from albumen. This phenomenon of transfer of water from the albumen to the yolk was explained by Greenlee (1911) by a simple process of osmosis. By osmosis the water passes through vitel-

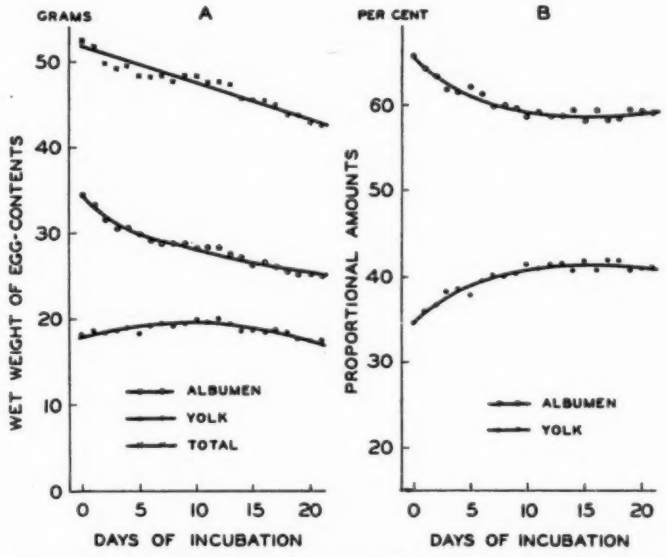

FIG. 2. Daily changes in the volume of egg contents with incubation. A, wet weights; and B, proportional amounts of yolk and albumen.

line membrane from the more dilute albumen to the more concentrated solution of yolk. Straub and Hoogerduyn (1929) showed that eggs stored for a long time tend to acquire equal osmotic pressure on both sides of the membrane.

Specific Gravity: Specific gravity of yolk rapidly fell down at the beginning of incubation and thereafter remained almost at the same value (Fig. 4-A). Specific gravity of albumen rapidly increased especially during the early part of incubation. The specific gravity of the outer fluid portion of albumen rose quickly during the first few days, and then it was closely parallel with and slightly below the values of the other layers of albumen, Romanoff and Sullivan

(1937). In both fluid and dense portions of albumen the specific gravities would correspond to their dry-matter content, Romanoff (1929a).

Index of Refraction: The refractive index of yolk was the highest in a fresh egg. There was a slight decrease in its value with incubation. The refractive index of various layers of albumen (Fig. 4-B) had undergone rapid changes towards high values. The trend of changes was similar to that observed at low temperature of 20°C.—Romanoff and Sullivan (1937)—but at a much more rapid rate. On

TABLE 4

Changes in Chemical Composition of an Unfertilized Egg During 21 Days of Incubation

Observations	A fresh egg		An incubated egg		Change in amount
	Contents	Dry basis	Contents	Dry basis	
	gm.	*pct.*	*gm.*	*pct.*	*pct.*
Ether extract:					
Yolk	5.367	53.35	3.882	51.55	— 27.67
Albumen	0.122	0.03	0.311	0.07	+154.92
Total	5.489	39.54	4.193	35.03	— 23.61
Saponification number	190.69		184.97		— 3.00
Iodine value	73.89		73.79		— 0.14
Refractive index	1.4685		1.4706		+ 0.14
Total nitrogen:					
Yolk	0.469	4.98	0.362	4.81	— 22.81
Albumen	0.493	13.50	0.587	13.22	+ 19.07
Total	0.962	6.93	0.949	7.93	— 1.35
Total ash:					
Yolk	0.2879	2.86	0.2673	3.55	— 7.16
Albumen	0.0797	2.09	0.0823	1.85	+ 3.26
Total	0.3676	2.65	0.3496	2.75	— 4.90
Calcium (as CaO):					
Yolk	0.0279	0.28	0.0276	0.37	— 1.08
Albumen	0.0059	0.15	0.0058	0.13	— 1.69
Total	0.0338	0.24	0.0334	0.28	— 1.18

the 21st day the average value at 38°C.(100.4°F.) reached to 1.3635, while at 20°C.(68°F.) only to 1.3590. Also at the incubating temperature all layers of albumen—outer fluid, middle dense, and middle fluid—after a few days became disintegrated and did not show appreciable differences in refractive index.

Hydrogen-Ion Concentration: The hydrogen-ion concentration of egg albumen from the original value of fresh eggs of about 7.8 rose rapidly within two to three days to 9.5 (Fig. 5-A). This rapid change has been attributed to the loss of carbon dioxide, Sharp (1929). Thereafter the high value of pH of albumen had remained during three weeks almost unchanged, which is in accordance with the pre-

vious observations of Sharp and Powell (1931) and Romanoff (1929, 1933). Earlier studies of Aggazzotti (1913) and others, however, indicate that if infertile eggs are incubated, there is no change from low value to high and that the value of pH of albumen is high from the beginning. The discrepancy in these observations was presumably due to the use of well-aged eggs for incubation, the eggs that already had high value of pH.

The hydrogen-ion concentration of yolk also changed but more slowly than that of albumen. The pH value of 5.9 of fresh yolks

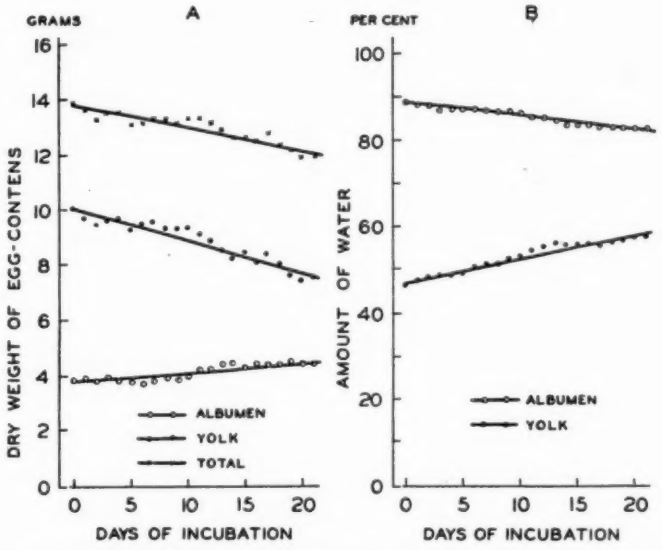

FIG. 3. Daily changes in dry weight and presence of water in egg contents with incubation. A, dry weights; and B, percentages of water in yolk and albumen.

gradually rose to 6.9 on the 21st day. The increase of pH of yolk presumably was due to a loss of carbon dioxide from the yolk to the albumen which in turn loses it to the surrounding air, Sharp (1929).

Content of Ammonia Nitrogen: The ammounia nitrogen content of fresh eggs was very low—.24 mg. per 100 c.c. of wet albumen and .99 mg. per 100 c.c. of wet yolk. During the 21 days of incubation the amounts of ammonia nitrogen rose to 1.26 mg. and 12.94 mg. per 100 c.c. of albumen and yolk, respectively (Fig. 5-B). The increase of ammonia nitrogen in both albumen and yolk was almost lineal, and the trend of increase was about the same as was observed

by Pennington and Robertson (1912), Hendrickson and Swan (1918), and Jenkins, Hepburn, Swan, and Sherwood (1920).

After three weeks of incubation there was about ten times more ammonia nitrogen in yolk than in albumen per volume. From this it is evident that chemical decomposition of proteins is more rapid in yolk than in albumen. Moreover, it is also possible that some of the ammonia nitrogen recovered from the albumen passed through vitelline membrane from the yolk to the albumen.

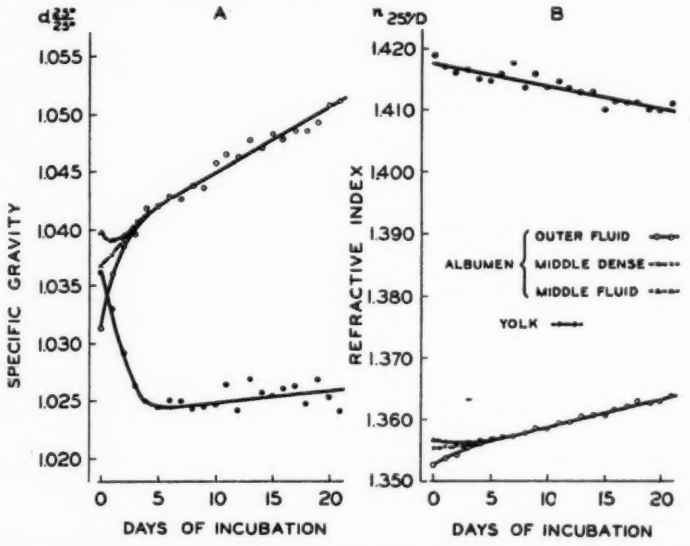

FIG. 4. Daily changes in egg contents with incubation. A, specific gravity; and B, refractive index.

Elasticity of Vitelline Membrane: There is a very large difference in osmotic pressure between yolk and albumen—about .45° △ of albumen and .60° △ of yolk of a fresh egg—which tends to equalize on both sides of the vitelline membrane, Straub and Hoogerduyn (1929). The volume occupied by the yolk is therefore increasing by inflow of water from the albumen. The diffusion of water into yolk through the vitelline membrane begins immediately after ovulation, that is, as soon as it comes into contact with the albumen in the oviduct, Bialaszewicz (1912) and Romanoff (1931). After the egg is laid, this process is continuous as long as the elastic membrane can stand the stretching.

During the incubation the vitelline membrane becomes more extensible. From the measurement of elasticity of the vitelline mem-

brane of these eggs it was observed (Table 3) that the membrane becomes quite stretched and very weak and is easily ruptured after one week of incubation. Approximately the same length of incubation at 37°C.(98.6°F.) was required for the membrane to rupture in the experiments of Sharp and Powell (1930) except that their index of elasticity—the ratio of the height divided by the width of the yolk laid on a plain surface—was much higher throughout than in our measurements.

Chemical Changes: The two chief chemical constituents of egg contents, aside from water, are fat and protein. The main chemical

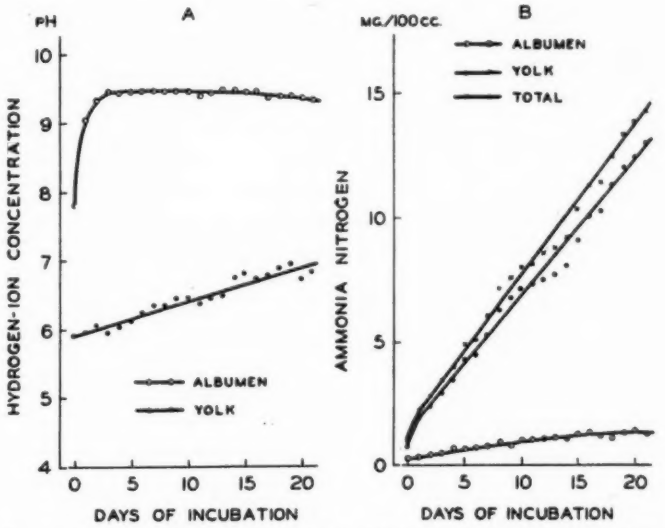

FIG. 5. Daily changes in egg contents with incubation. A, hydrogen-ion concentration; and B, amount of ammonia nitrogen.

changes within the incubated eggs, therefore, would be the volatilization of fatty acids and denaturation of proteins from the molecular to the micellar state—Loughlin and Lewis (1932)—with the increase in the number of —SH group. It also was shown by Pennington and Robertson (1912) that enzymatic activity plays an important rôle in organic decomposition of unfertilized incubated eggs.

The chemical analyses of fresh and 21-day incubated eggs (Table 4) show that yolk lost while albumen gained in both ether extract and total nitrogen. Evidently fat and protein derivatives as well as glucose—Pucher (1928)—diffuse through the vitelline membrane from the yolk into the albumen. At the same time some volatile or gaseous

substances escape from the albumen through the pores of the shell. Consequently, the total amount of ether extract and total nitrogen diminished in the egg.

The nature of yolk fat was found to be practically unchanged. There was no appreciable increase in the free fatty acids. The measurements of saponification number, iodine value, and refractive index gave but very slight changes. Similarly, Thomson and Sorley (1924) observed that the yolk of an egg, tested one hour after being laid, showed the presence of 1.72 per cent of free fatty acids (stated as oleic acid) in the fat, while after three months of storage the amount of free fatty acids is very little increased. Only after one year this figure rises to 3.12 per cent and at the end of two years to 5.15 per cent.

There was found proportionally more ash in incubated eggs, especially in yolk, than in fresh eggs. Similar phenomenon was observed by Willey, *et al.* (1908) in cold-storage eggs.

COMPARISON OF INCUBATED WITH COLD-STORAGE EGGS

Since there is a rapidly growing interest in the early detection and removal from the incubator and the economic use of unfertilized eggs, especially for human food, it is interesting to compare the rate of decomposition of eggs at 37.5 to 38°C.(99.5 to 100.4°F.) with those that are kept in commercial cold storage at 0 to 2°C.(32 to 35.6°F.).

It was observed that there is about five per cent loss in weight of eggs held in cold storage for seven—Willey, *et al.* (1908)—to nine months—Jenkins and Pennington (1919)—although at somewhat higher humidity. This loss would be equivalent to that of the eggs incubated less than one week (Fig. 2-A). During nine months of storage there is about two per cent loss of water in albumen and about three per cent gain of water in yolk—Jenkins and Pennington (1919). These amounts in loss of water from albumen and in gain of water in yolk by the process of osmosis can be observed in eggs incubated not over one week (Fig. 3-B). The amount of ammonia nitrogen in mixed samples of cold-storage eggs, including albumen and yolk, was found to be gradually increasing to about 4 mg. per 100 c.c. on the seventh to ninth month of cold storage—Jenkins and Pennington (1919) and Lythgoe (1927). Comparing these values with those in Fig. 5-B it is evident that seven to nine months of cold storage would be equivalent to about one week of incubation. It also has been shown by Sharp and Powell (1930) that in cold storage the vitelline membrane is weakened but separation of yolk from albumen is possible after 11 months—Jenkins and Pennington (1919).

In incubated eggs after eight days of incubation the vitelline membrane invariably ruptures when the egg is opened (Table 3). The changes in pH of yolk and of albumen of storage eggs—Sharp and Powell (1931)—are comparable with the incubated eggs (Fig. 5-A) in nearly similar time-ratio.

The above evidence would indicate that in respect to chemical decomposition, one day of incubation of unfertilized eggs is equivalent to about five to six weeks of their cold storage. If the rate of decomposition is directly proportional to temperature—Sharp (1929), Smith (1930), and Straub and Hoogerduyn (1929)—then it would seem that for each degree of holding temperature above 0°C. there should be added approximately one day extra for aging. For example, 20 days of storage at 0°C. would be equivalent to one day of storage at 20°C., and 38 days of storage at 0°C. to one day of incubation at 38°C. Therefore, in order to preserve the original freshness of the egg as long as possible, the egg should be put into a cool room immediately after being laid.

DISCUSSION

Chemical instability is one of the properties of birds' eggs. Immediately after being laid, the whole physicochemical system of the egg undergoes gradual and continuous changes from a virgin, fresh state into a stale, superinduced one.

A strictly fresh egg displays a very small air cell, which results from the contraction of the contents with cooling. At this stage the yolk has a slightly higher density than albumen; therefore, the yolk occupies nearly a central position in the egg. Both yolk and albumen have the lowest acidity values shown by hydrogen-ion concentration, although the difference in osmotic pressure between yolk and albumen is very high.

With aging the air-cell increases—Romanoff (1930)—indicating the reduction in volume of egg contents. The main internal change is the loss of water through the shell in the process of evaporation and the movement of water from albumen to yolk and back after reaching the osmotic equilibrium. Yolk soon becomes less dense and floats up in a more dense albumen. The increased volume of yolk in the process of the absorption of water considerably weakens the elasticity of vitelline membrane, which then can be easily ruptured.

In the course of chemical changes within the egg during aging or incubation the simple organic constituents, resulting from molecular disintegration, move from yolk to albumen. Among these are glucose, fatty acids, and simple proteins. During this process the dry matter of yolk decreases while that of albumen increases. But

as the result of further chemical disintegration, especially of yolk, there is noticeable escape through the albuminous layer and through the eggshell of some volatile substances, vapors, and gases, such as volatile oils, water vapor, carbon dioxide, ammonia, and possibly hydrogen sulphide. In spite of the fact that there is evident proportional increase of ash and calcium, there is a marked decrease in total dry-matter content with the age of the egg. Furthermore, the absorption of oxygen and the elimination of heat bring further evidence of the presence of progressive chemical disintegration.

On the whole, with the aging of the egg there is observed not only the loss in weight but also pronounced chemical decomposition and decrease in dry-matter content. This would lower the quantitative value of eggs and decrease their beneficial biologic and prophylactic effects when the eggs are used for human consumption.

SUMMARY

It took about 10 months to dehydrate the whole egg at 20°C., and a little over four months at 37.5°C.

The average daily loss in weight of a 60-gram egg, incubated at 38°C. and 60 per cent of relative humidity, was .446 gram. The change in daily loss of .01 gram per egg could be brought about by either .6 per cent relative humidity, or 1.9°C., or 2.6 feet per second air movement, or 3 c.c./1 cm.^{2}/1 min. permeability of shell to air, or six grams of egg weight.

During incubation the volume of albumen was diminishing, while that of yolk at first was increasing then decreasing, owing to the flow of water from albumen to yolk and back by the process of osmosis. This was accompanied by noticeable changes in specific gravity, refractive index, and hydrogen-ion concentration in both yolk and albumen.

The dry matter of yolk decreased and that of albumen increased because of diffusion of glucose, fatty acids, and simple proteins from yolk to albumen. As the result of chemical decomposition and then of escape through the eggshell of volatile oils, water, and carbon dioxide, there was found less total nitrogen and ether extract in the egg contents at the end of 21 days of incubation, but proportionally more calcium than in a fresh egg.

In respect to chemical decomposition, one day of incubation of unfertilized eggs is equivalent to about five to six weeks of their cold storage; or for each degree of holding temperature above 0°C. there should be added approximately one day for aging.

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SCORING OF TOAST AND FACTORS WHICH AFFECT ITS QUALITY [1]

W. H. CATHCART AND E. J. KILLEN
Research Department, American Institute of Baking, Chicago, Illinois

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In a recent survey by Mankertz (1939) of persons living in the city of Seattle, Washington, and vicinity it was shown that the average individual consumes about 35 to 40 per cent of his bread in the form of toast (a high percentage questioned by some workers). Indications are that this figure would hold fairly well for the country as a whole, although surveys are not available that give figures for other parts of the United States. Why is it that so much bread is consumed in the form of toast?

The aroma, appearance, and anticipated taste of a food that is about to be consumed has a great deal to do with the amount that is eaten. These are not the only factors, for as the food is being eaten, texture and taste come into play. As pointed out by Crocker and Platt (1937), I. P. Pavlov showed that pleasant anticipation (experiments on dogs) caused digestive juices to flow. Research by physiologists and psychologists have shown that in order for food to be eaten in quantity and digested properly it must be attractive. In order for a food to be consumed in quantity it must have a pleasing and satisfying effect on the nerve ends that detect odors and on the taste buds.

When bread is toasted, some of the starch is dextrinized, some of the sugars are caramelized, and some of the proteins are parched. It is believed that these chemical reactions are the basis for the attractiveness of good toast. The taste of toast undoubtedly is a blend between the primary tastes, that is, sweet, sour, salty, and bitter; and to most people of all ages it is a pleasing blend. The chemical constituents that make up the combined odor have not been determined. This latter field would undoubtedly be worthy of research.

Good toast has an appetizing, golden brown color; a smooth, crunchy, tender, toasted surface; a tender crumb underneath the toasted surface; and a pleasant, sweet, appetizing odor and taste.

Previous workers [2] commented that the nutritive value of bread is probably not increased by toasting, but its palatability and digestibility are greatly increased. The American Institute of Baking did

[1] Summary read before the Boston meeting of the American Chemical Society, September, 1939.

[2] Reports presented in "Baking Technology" 2, 102 (1923).

a considerable amount of research in connection with the national toast campaign that was sponsored by the Institute and the Wheat Council of the United States.[3] One result of this research was the development of better toasters—toasters that would handle slices of bread sold on the American market and toast bread in the right manner.

At the time this research was done the standard toast was taken to be a compromise between two extremes, that is, a slice where the outside was browned very quickly and a slice that was browned all the way through.[2] It was found then that five to six minutes[4] were required to turn out the best toast (using an electrical toaster). Toast made in three minutes in front of a very hot surface suffered in quality. The results of this work showed that good bread in general made good toast and bad bread made bad toast. Bread from 18 to 30 hours old made the best toast.

Since this pioneer work was done on toast, about 15 years have passed. With that passage of time changes have been made. Consumers have developed certain likes and dislikes which were not prevalent 15 years ago. Toasters have changed. The toaster which is today found in the average home does not require five to six minutes to toast a slice of bread. After the first slice has been toasted and the toaster is warm, subsequent slices are toasted in one to two minutes. It has been determined that about 10 to 12 watt hours (34 to 40 B.T.U.) are required to toast a slice of bread in a modern toaster.[5] This quicker toasting time gives a different type of toast than would be obtained in five to six minutes. Undoubtedly several things have brought about this change in toasting time: modern trends are toward the shortening of time for performing any given task; bread today contains more sugar and milk than it did fifteen years ago; and perhaps the public has learned to prefer or does prefer this newer type of toast. The standard toast score sheet that was used for this pioneering research work is given (Table 1).

At the American Institute School of Baking, products from the experimental bakery are scored for their toasting quality (score in Table 1 used) as well as bread quality. The summary of the results obtained throughout the past 15 years is quite interesting. Results[6] are as follows:

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[3] Reports presented in a series of articles in "Baking Technology" (1922 to 1927).

[4] For toasters that toasted only one side at a time. Minutes given are total toasting time.

[5] Data obtained from the McGraw Electric Company.

[6] Summary of these results is on file at the Louis Livingston Library of Baking, American Institute of Baking, Chicago, Illinois.

1. Bread with normal [7] per cent sugar (sucrose) in the formula gives best toast. High percentage of sugar gives better toast than low percentage of sugar.

2. Bread with the same percentage of different kinds of sugar gives about the same quality toast.

3. The use of diastatic malt in bread will improve the toast score.

4. Bread with normal [7] percentage of yeast gives best toast. Lower percentages give better toast than higher percentages of yeast.

5. Bread with normal [7] percentage of salt gives best toast. Excess is better than none at all.

6. Dry skim milk in formula up to three per cent improves toasting quality. Above this, it is better than no milk.

TABLE 1

Toast Score First Used in Research at American Institute of Baking

	Ideal
Time (minutes)	8
Color	10
Thickness of crust	15
Character of crumb	25
Flavor	25
Taste	25
Total	100

7. Four per cent of powdered whole milk in formula gives best toast. A higher percentage gives better toast than no milk.

8. Increasing the percentage (actual amounts not given) of sweetened condensed milk in formula gives better toast score.

9. Using about 60 per cent of whole milk to replace liquid in formula gives best score. Full milk bread gives better score than water bread.

10. Toast score was approximately the same when supplying full milk in various forms to this formula.

11. Increasing shortening up to about three per cent in formula increases the toast score.

12. Varying the kinds of shortening in formula varies the toast score. Solid shortenings give better score than liquid.

13. The toast score drops as the percentage of clear flour is increased in the flour blend.

14. Normal dough temperature, 25.6 to 26.7°C. (78 to 80°F.), gives best toast score. Cool dough gives better toast than warm dough.

[7] Exact percentage not given. All percentages on basis of flour 100 per cent.

15. Normal punch gives best toast score. Frequent punch is better than no punch.

16. Overproofed loaves score less than underproofed loaves.

17. Normal dough consistency gives best toast score. Stiff doughs are a little better than slack doughs.

18. An old dough scores less than a young dough.

It must be remembered that these results were obtained by students during a regular scoring class and they represent the average of the work of many different classes.

Darnell (1937), in connection with the Bread Consumption Committee of the American Society of Bakery Engineers, has stated that there is a vast difference in the toasting quality of bread, and from some questionnaires he gave opinions as to how to make good toast. One interesting point which was brought out was that the ordinary

TABLE 2

Percentage Preferences on Round-Top, Twisted, and Sliced Bread

Type of bread	Preference
	pct.
Round top	90
Sandwich	10
Twisted	0
Not twisted	100
Sliced	35
Not sliced	65

sandwich-type bread makes "the poorest toast that possibly can be made." It is stated that it is tough and does not have the flavor desirable.

Mankertz (1939) reported work from a survey (details of consumers canvassed not given) which showed that medium brown was preferred either to a dark brown or to a very light toast and that round-top bread was preferred to any other type of bread for toast. From actual tests with a jury of 10 men and women a rich formula was found to be slightly preferred to a lean formula for toast. The figures presented on round-top, twisted, and sliced bread are of sufficient interest to be presented (Table 2).

EXPERIMENTAL WORK

Since there is a great deal of bread eaten as toast, a means of scoring the toasting quality of bread would be desirable. It would undoubtedly prove valuable to handle this in a similar manner to the way bread scoring is done today; that is, score the loaf for toast-

ing value. The primary purpose of this work was to develop a toast score that could be used for this purpose; the first score worked with (Table 1) was used during the American Institute of Baking toast campaign. After many experiments were made, certain changes suggested themselves and resulted in the score given (Table 3).

Several different toasters were tried, but it was finally decided that since the type of toaster affects the toast and most bread is toasted in homes, a toaster should be selected that would be comparable to what the housewife uses. Thus, the heavy duty restaurant machine was not used. The type finally decided upon was a popular, automatic, well-made, household toaster.

TABLE 3

Tentative Toast Score

Loss in weight during toasting of two slices.....	
	pct.
Color	20
Character of crust	10
Character of toasted surface	15
Character of crumb	15
Aroma	20
Taste	20
Total	100

It is realized that all people do not like the same kind of toast; preferences range from a soft, crisp, crusted slice to a hard, dry slice which has been toasted through and through. According to the authors' observations, however, modern toasters will not make the hard, dry type of toast. During the early research the American Institute of Baking recommended that the toasted surface of the bread should be one-third the entire thickness of the slice, thus leaving only one-third of the original bread on the inside. Modern toasters will not do this; they produce a much thinner toasted surface.

The authors have found that a good loaf of bread, whether from a lean or a rich formula, will make a very good piece of toast, provided the amount of heat and the time of toasting are varied. The average housewife, however, is not going to vary the amount of heat and the time of toasting except within small limits. Since the type adopted was a household-type toaster, no means was provided for varying the amount of heat and no attempt was made to do it. The toaster did have a time regulator and this was adjusted to what was considered the optimum length of time for toasting the average pan bread found on the Chicago market (color of the toast was the main

indicator). The optimum time setting gave a toasting time which varied from one and one-fourth to one and one-half minutes (determined after the toaster was hot). Once this optimum was established, bread from different formulas was used and the time proved satisfactory over quite a variation in range of formulas. A time element greater than that chosen seemed to almost always burn the toast (carbonize the toast) no matter what the formula. Times shorter than this always seemed to give a light toast, no matter whether or not a very rich formula was used.

When determining the toasting time, different thicknesses of slices from the same loaf of bread were tried. Variations from three-eighths to five-eighths of an inch did not require different toasting times, the

TABLE 4

Basic Formula

All ingredients based on flour as 100 per cent	
	pct.
Flour	100
Water	Variable
Salt	2
Yeast	2
Sugar	4
Milk	4
Shortening	3
Yeast food	.25
Malt extract	1

same time being required to produce optimum toast from any thickness between these two.

In all the remaining experiments the toaster was allowed to heat one and one-half minutes before toasting and all toast was made with the optimum time setting. The bread was sliced one-half inch thick on a mechanical slicer just before toasting. The individual loaves were made under controlled laboratory conditions and were stored in a commercial manner before toasting.

Having decided upon a tentative score, the toaster, and optimum toasting time, it was next thought advisable to try various percentages (all percentages on basis of flour as 100 per cent) of different ingredients in a bread formula and attempt to determine their effects on the resulting toasting qualities of the loaf. One ingredient was varied in any one particular day's experiment; the resulting bread was toasted and scored (24 hours after baking) according to the tentative score. Mixing temperatures, punching, proofing, absorption, fermentation time, methods of make-up, and age before toasting were

also studied. In this way the effect upon each factor for which the bread was to be scored could be studied. Factors being affected could be retained in the score and those not being affected could be eliminated. The basic formula (high-grade patent flour used) is given (Table 4). The amount of only one ingredient was varied in one day's experiment, unless otherwise stated. The rest of the formula remained as represented (Table 4); thus, any variation in the toast score was due to the one variable allowed during that one particular set of experiments. The data obtained by this procedure (Table 5) show that comparison of results should be limited to each individual day's experiments; results on different days should not be compared owing to the possible error of scoring after the lapse of time. Also, an effort was made to show differences between the individual experiments in one day's run.

Unless otherwise stated, the experiments were not run in duplicate, for, as will be seen below, they were on the whole in very good agreement with the A.I.B. School experiments. Also a few experiments indicated that the average technician could repeat his findings on replicate samples prepared during the same day.

DISCUSSION OF RESULTS

Day 1 (Table 5) shows that as the percentage of sucrose is increased, the toast score increases. The results of Day 2 on sucrose show that this increases up to 10 per cent and perhaps above. The results of Day 8 also show that bread made with sucrose scores slightly higher than bread made with the same amount of dextrose. These results are not quite in keeping with the summary given of the experimental bakery results at the A.I.B. since the normal percentage of sugar stated to give the best toast is not as high as 10 per cent.

Days 3 and 4 show that diastatic malt extract slightly increases the toast score, which agrees with the A.I.B. results. The optimum score is with a higher percentage of malt than is normally used in commercial practice. It should be pointed out here that the flour used for these experiments was a very strong, high-grade flour and was well balanced enzymatically. A weaker flour would perhaps give optimum results with a lower percentage of diastatic malt extract.

From Day 5 it is seen that varying the percentage of yeast up to four per cent had little effect on the toast score. The one experiment with five per cent of yeast seemed to fall off as far as score was concerned. These results more or less verify those of the students' experiments in the American Institute School of Baking.

TABLE 5

Toast Score of Bread Made by Varying the Factors as Stated[1]

Day	Factor tested	Per cent of ingredient (on basis of flour, 100%)	Color	Character of crust	Character of toasted surface	Character of crumb	Aroma	Taste	Total	Loss in weight during toasting of two slices
			20	10	15	15	20	20	100	*pct.*
1	Sucrose	0	15.0	8.0	12.0	14.0	15.0	15.0	79.0	11.1
		2	16.0	8.0	13.0	14.0	16.0	16.0	83.0	12.0
		4	18.0	8.5	13.0	14.0	18.0	18.0	89.5	13.7
		6	18.5	8.5	13.0	14.0	18.5	19.0	91.5	13.7
		8	19.0	8.5	13.5	13.5	18.5	19.5	92.0	13.5
2	Dextrose	8	16.5	8.5	13.5	13.5	18.5	19.0	89.5	13.6
		10	17.0	9.0	13.5	13.0	18.5	19.0	90.0	14.6
	Sucrose	8	18.5	9.0	13.5	13.0	19.0	19.5	92.5	16.7
		10	19.0	9.0	13.5	12.5	19.5	20.0	93.5	15.4
3	20° Lintner malt	0	17.5	8.5	13.5	13.0	18.5	18.5	89.5	15.4
		1	18.0	8.5	13.5	13.0	18.5	18.5	90.0	16.5
		2	18.5	8.5	14.0	13.0	19.0	19.0	92.0	19.5
		3	18.0	8.0	13.5	13.0	19.0	19.0	90.5	18.4
		4	17.0	8.0	13.5	13.0	18.5	18.0	88.0	19.4
		5	16.5	8.0	14.0	13.0	18.0	18.0	87.5	15.6
4	60° Lintner malt	0	17.0	8.0	13.5	13.5	18.5	18.5	89.0	13.2
		1	17.5	8.0	13.5	13.5	18.5	18.5	89.5	16.3
		2	18.5	8.0	13.5	13.0	19.0	19.0	90.0	15.7
		3	19.0	8.0	13.5	13.0	19.0	19.0	91.5	15.0
		4	18.5	8.0	14.0	13.0	18.5	18.5	90.5	13.5
5	Yeast	1	17.0	8.5	14.0	13.5	18.5	18.5	90.0	15.2
		2	17.5	8.5	13.5	13.5	19.0	18.5	90.5	15.0
		3	18.5	8.5	13.5	13.5	19.0	18.5	91.5	14.5
		4	18.0	8.5	13.5	13.5	18.0	18.0	89.5	16.5
		5	17.5	8.5	13.5	13.5	17.5	18.0	88.5	17.5
6	Salt	0	18.0	8.0	13.5	13.5	17.0	17.0	87.0	15.4
		1	17.5	8.0	13.0	13.5	18.5	17.5	88.0	16.0
		2	18.0	8.0	13.5	13.5	18.5	18.5	90.0	15.2
		3	15.0	8.5	14.5	13.5	18.0	17.0	86.5	16.7

[1] The factor tested was the only variable allowed during any one day's experiment.

TABLE 5 *(Continued)*

7	Dry skim milk	0	16.0	8.0	13.0	13.5	17.0	17.0	84.5	15.6
		2	16.5	8.0	13.0	13.5	17.5	17.0	85.5	14.0
		4	18.0	8.5	13.5	13.0	19.0	19.0	91.0	13.5
		6	19.5	8.5	13.5	13.0	19.5	19.5	93.5	17.4
		8	20.0	8.5	13.5	13.0	20.0	20.0	94.0	19.2
8	Dry whole milk	0	16.0	7.5	12.5	13.5	16.5	18.0	84.0	12.3
		2	16.0	8.0	12.5	13.0	17.0	18.0	84.5	15.0
		4	18.5	8.5	13.0	12.5	17.5	18.5	88.5	13.9
		6	18.0	9.0	13.5	12.0	18.5	19.0	90.0	12.5
		8	18.0	9.5	13.5	12.0	19.0	19.0	91.0	18.4
		10	18.0	9.5	13.5	12.0	19.5	19.0	91.5	18.5
9	Liquid whole milk	0 Per cent of total liquid	17.0	8.0	13.0	14.0	16.0	18.0	86.0	16.8
		20 Per cent of total liquid	17.0	8.5	13.0	14.0	16.5	18.0	87.0	15.7
		40 Per cent of total liquid	17.5	8.5	13.0	13.5	18.0	19.0	89.5	16.5
		60 Per cent of total liquid	18.5	9.0	13.0	13.5	19.0	19.0	92.0	15.8
		80 Per cent of total liquid	19.0	9.5	13.0	13.0	19.0	19.5	93.0	16.3
		100 Per cent of total liquid	18.5	10.0	13.0	13.0	19.5	19.5	93.5	15.7
	Dry whole milk	9	18.5	10.0	13.5	13.0	19.5	19.5	94.0	15.0
10	Fully hydrogenated vegetable shortening	0	16.5	7.5	13.5	13.0	18.0	18.0	86.5	16.7
		2	17.0	8.0	13.5	13.5	18.5	19.0	89.5	17.6
		4	18.5	8.0	13.5	13.5	19.0	19.0	91.5	21.8
		6	18.5	8.0	13.5	13.5	19.0	19.0	91.5	20.0
11	Various fats and oils	4—Corn emulsion	18.5	8.0	13.5	13.5	19.0	19.0	91.5	17.0
		4—Hydrogenated	18.0	8.5	13.0	13.0	19.0	19.0	90.5	18.0
		4—Lard	18.0	8.5	13.0	13.0	19.0	19.0	90.5	16.7
		4—Puff paste	19.0	8.5	13.5	13.0	19.0	19.0	92.0	.....
		4—Butter	17.5	8.5	13.5	13.0	19.0	19.0	90.5	14.0
		4—Cottonseed oil	18.5	8.5	13.5	13.0	19.0	19.0	91.5	15.2
		4—Vegetable oil	18.5	8.5	14.0	13.0	19.0	19.0	92.0	14.8
		4—Corn oil	18.5	8.5	13.5	13.0	19.0	19.0	91.5	16.4
12	Clear flour in blend	0 Per cent in flour blend	19.0	9.0	13.0	14.0	18.5	19.0	92.5	15.2
		20 Per cent in flour blend	18.5	9.0	13.0	14.0	18.5	19.0	92.0	16.0
		40 Per cent in flour blend	18.0	9.0	13.5	14.0	18.5	19.0	92.0	18.1
		60 Per cent in flour blend	17.5	9.0	13.5	14.0	18.5	19.0	91.5	18.0
		80 Per cent in flour blend	17.0	9.0	13.5	14.0	17.5	18.5	89.5	17.8
		100 Per cent in flour blend	17.0	9.0	13.5	14.0	17.0	18.5	89.0	.....

TABLE 5 *(Concluded)*

Day	Factor tested	Per cent of ingredient (on basis of flour, 100%)	Color	Character of crust	Character of toasted surface	Character of crumb	Aroma	Taste	Total	Loss in weight during toasting of two slices
			20	10	15	15	20	20	100	*pct.*
13	Temperature mixed	27.2°C.(81°F.)	18.0	9.0	14.0	13.0	19.0	19.0	92.0	17.5
		30.6°C.(87°F.)	18.0	9.0	14.0	13.0	19.0	19.0	92.0	17.1
		35.6°C.(96°F.)	18.0	8.5	13.5	13.0	19.0	19.0	91.0	18.5
14	Punching	Normal	18.5	9.0	13.5	13.0	19.0	19.0	92.0	18.0
		No punch	18.0	9.0	13.0	13.0	19.0	19.0	91.0	20.0
		Every 20 min.	18.0	9.0	13.0	13.0	19.0	19.0	91.0	19.7
15	Proofing	Overproofed	17.9	9.0	12.5	13.5	18.5	19.0	89.5	19.2
		Underproofed	18.5	9.0	13.5	13.5	19.0	19.0	92.5	17.5
	Absorption	Slack	18.5	9.0	14.0	13.0	19.0	19.0	92.5	17.7
		Stiff	19.0	9.0	14.0	13.5	19.0	19.0	93.5	16.5
	Fermentation time	Young dough	17.0	8.5	12.0	13.0	18.5	19.0	88.0	15.1
		Old dough	19.0	9.0	13.5	13.0	19.0	19.0	92.5	15.7
		Normal dough	19.0	9.0	14.0	13.5	19.0	19.0	93.5	22.0
16	Make-up	Round-top	18.5	9.5	13.5	13.5	18.5	19.0	92.5	14.6
		Twist	18.0	9.0	13.0	13.0	18.5	19.0	90.0	19.3
		Cross-pan	19.0	9.5	13.5	13.5	18.5	19.0	93.0	15.6
17	Make-up	Round-top	18.0	9.0	13.0	13.5	19.0	19.0	91.5	14.0
		Twist	18.5	9.0	14.0	13.0	19.0	19.0	92.5	15.2
		Pullman	18.5	9.0	13.5	13.0	19.0	19.0	92.0	14.4
18	Age of bread before toasting	*hr.*								
		2½	17.5	9.0	13.5	13.0	18.5	19.0	91.0	17.5
		4	18.0	9.5	13.5	13.0	18.5	19.0	91.5	16.3
		8	18.0	9.5	13.5	13.0	18.5	19.0	91.5	16.4
		14	18.0	9.5	13.5	13.0	18.5	19.0	91.5	22.0
		24	17.5	9.5	13.0	13.0	18.5	19.0	90.5	19.1
		36	17.5	9.5	13.0	13.5	18.5	19.0	91.0	17.5
		48	18.0	9.5	13.5	13.5	18.5	19.0	92.0	17.3
		72	18.0	9.5	13.5	13.5	18.5	19.0	92.0	15.7
		98	18.0	9.5	13.5	14.0	18.5	19.0	92.5	26.0

The experiments from Day 6 on salt fall in line with those of the A.I.B. experiments in that normal percentages (two per cent) of salt give the best toast.

The experiments from the School of Baking indicate that three per cent powdered skim milk, four per cent powdered whole milk, and 60 per cent whole raw milk (40 per cent water in dough) individually give the best toast. The results from Days 7, 8, and 9 (Table 5) show that the toast score increased as the percentage of milk increased no matter what kind was used. The highest percentage of milk of the various kinds tried gave the best result. These highest percentages were as follows: eight per cent dry skim milk; 10 per cent dry whole milk; and 100 per cent (of total liquid; milk bread) liquid whole milk. The loaf with nine per cent dry whole milk included in Day 9, along with the liquid whole-milk experiment shows that either dry whole milk or liquid whole milk to make milk bread produced equally good results. The results agree with the School of Baking experiments in that the toast score was about the same when supplying whole milk in various forms.

Day 10 shows that increasing the amount of shortening increases the toast score; however, above four per cent no increase was noted. Percentages of shortening above six per cent were not tried. This is in agreement with the School of Baking experiment.

The experiments from Day 11 do not agree with the conclusions as given from the School of Baking experiments; in the latter it was concluded that varying the kinds of shortening varies the toast score. In the experiments of Day 11, four per cent of various types of shortening were used and although the final toast score does vary slightly, the variations were not significant.

The experiments in Day 12 show that as the percentage of clear flour increases in the blend, the resulting toast score drops. This is in agreement with the results of the School experiments.

The results of Day 13 show that different temperatures at which the dough is mixed make little difference in the final toast score. The loaves, of course, were fermented at normal temperatures. This is hardly in accord with the results of the School experiments.

From Day 14 it is seen that the dough with the normal number of punches scored slightly the highest, which is in accord with the School experiments. There was no difference between a no-punch dough and one punched every 20 minutes.

The results of Day 15 show that overproofed doughs scored less than underproofed doughs, stiff doughs gave a little better toast than slack doughs, and slightly old doughs gave better toast than slightly young doughs. The two former results are in agreement with the

results of the School's experiments, and it should be pointed out that the normal dough run along with these experiments of Day 15 produced the best toast except for the stiff dough which scored the same.

The bread from Day 16 shows that twist bread produces poorer toast than round-top or cross-pan bread,[7] the latter two producing toast of about the same score. Owing to the fact that the quality of the slices of twist bread vary from the same loaf, this experiment was repeated with a Pullman (not from brake dough) loaf replacing the cross-pan loaf. In this experiment (Day 17) the twist and Pullman (sandwich)[8] were better than the round-top. Thus, the authors cannot agree with Darnell and Mankertz that twisted or sandwich loaves do not make desirable toast. The "character of crumb" of the round-top bread was slightly better on each day but the round-top loaves lost on other factors of the score.

Day 18 shows the score of toast made from the same bread at different ages. A different loaf was taken for each experiment. Although there is a slight tendency for the toast score to increase from bread at two and one-half to 98 hours old, it is undoubtedly not significant; and the toast made from bread four hours out of the oven is practically as good as at any other time. These results do not verify the recommendations of many authors that bread should be at least 24 hours old to make the best toast.

It is generally stated that the reason fresh bread will not make good toast is due to its higher moisture content. It is of interest to point out that bread, as wrapped today, loses very little moisture over a considerable period of time. Results by Cathcart and Pushnik (1939) on sliced, moisture-proof, wrapped bread show that from the time of wrapping until the bread is 72 hours old, only a two-per cent loss of moisture occurs. It is realized, however, that the slices must have uniform moisture distribution to produce uniform toast.

These experiments were run not only to determine the effect of various factors on toast quality but to see if the factors of the score would be affected by toasting the different loaves of bread. The factors which were affected would be important to have in the score; if any should not be affected, they could be eliminated from the score. A review of all the data (Table 5) shows that all of the factors of the toast score were affected and thus they undoubtedly have some place in the toast score. From the many tests which have been made the authors feel that all of these factors belong in the toast score. During the toasting experiments characteristics were noted for which

[8] Round-top, twist, and Pullman bread were all made from the same dough.

the toast could be penalized under each factor in the score. The important ones of these are set up with the final score and are given (Table 6).

The loss in weight on toasting two slices of the bread was included in the tentative score with the hope that it might yield some correlation with some of the factors of the score or perhaps the total score. A study of the results shows that there is no correlation between this percentage of moisture loss and the total score or the other factors of the score; thus, it has been eliminated from the final score (Table 6). The figures are presented (Table 5) only because they

TABLE 6

Toast Score

Factor	Maximum score	Sample score	Penalized for	
Color	20		Too dark	Too light
			Spotted	Streaked
			Not uniform	Dark edges
			Not "golden"	Dull
Character of crust (bread)	10		Hard	Thick
			Tough	Too brittle
Character of toasted surface	15		Too thin	Too thick
			Porous	Rough
			Not crisp	Tough
			Hard	Too brittle
Character of crumb	15		Doughy	Tough
			Wet	Stringy
			Too dry	Too thin
			Too thick	
Aroma	20		Burned protein odor	Lack of aroma
			Strong	Not appetizing
Taste	20		Strong	Flat
			Carbonized	
Total	100			

EXPLANATION OF FACTORS IN SCORE

Color: Both surfaces of the slice should toast uniformly to yield an appetizing, rich, golden brown color.

Character of crust (bread): After toasting, the crust of the slice should be thin and should break easily. It should not be thick and tough.

Character of toasted surface: The toasted surface should be tender, smooth, and crunchy. Considering modern bread and toasters, the depth of carameliaztion should be approximately 1/16 to 1/8 inch on each side. It should not be porous.

Character of crumb: The crumb should be tender and free from doughiness and stringiness. The crumb is obtained with a pointed instrument from between the two toasted surfaces. The thickness of the toasted surface is also obtained by this process.

Aroma: Toasting enhances aroma. The aroma of good toast should be pleasant, sweet, and appetizing. This is obtained while the toast is hot.

Taste: Good toast should have a pleasant taste, that is slightly sweet and suggestive of nuts.

do give information on the amount of moisture loss (plus other volatile substances) when bread is toasted.

Using the score given (Table 6), the following suggestions are made for scoring bread for toasting quality:

1. Toast two slices simultaneously.
2. Be sure toaster is hot.
3. Cut slices uniformly one-half inch thick unless the bread is already sliced. In this case, toast it as it is sliced.
4. Toast bread for same length of time in all cases.
5. Break one slice open and obtain the aroma while the toast is hot. Taste the same slice and note its mastication.
6. Examine the other slice for color, character of crust, character of toasted surface, and character of crumb.

The factors which affected the color of the toast are fermentation time, make-up, amount of clear flour in the blend, amount of shortening, amount of sugar, amount of malt, and amount of milk (Table 5). The amount of yeast and salt affected the various factors; however, they will not be considered here owing to the fact that bread quality limits the amount that can be used. The character of the crust was affected by the amount of milk, fermentation time, time of proofing and make-up. The character of the toasted surface was affected mainly by such factors as proofing, make-up, and fermentation time. The character of crumb was affected by the amount of milk and the age of bread before toasting. The aroma was affected by amount of sugar, amount of malt, amount of milk, amount of shortening, and amount of clear flour in the blend. The taste was affected by the amount of sugar, amount of malt, amount of milk, and amount of shortening. The way in which these factors are affected by the various processes or ingredients is evident from an examination of the table.

SUMMARY

In order to keep bread at its optimum for producing good toast, some comparative measure of its capability of making good toast is required. Thus, a toast score has been developed and is proposed for use in scoring bread for toasting quality. Its various factors have been examined by varying the percentages of various ingredients in making bread, varying methods of make-up and processing of dough, and then toasting the resulting bread with an automatic home-type toaster, using the score. The various factors in the score were found to vary with certain variations in the formula or process of making the bread. All of the factors in the score—color, character of crust (bread), character of toasted surface, character of crumb, aroma, and taste—are thought to be significant.

Factors which affect the quality of bread for toast have also been enumerated.

The toast of today is quite different from that made 10 or 15 years ago. This is due to the fact that formulas are richer, toasters are different, and people's tastes and likes have undoubtedly changed.

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FURTHER STUDIES ON DEVELOPMENT OF *CLOSTRIDIUM BOTULINUM* IN REFRIGERATED FOODS[1]

FRED W. TANNER, PARKER R. BEAMER, AND CHARLES J. RICKHER
Department of Bacteriology, University of Illinois, Urbana, Illinois

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INTRODUCTION

The history of botulism dates from early times. Symptoms of botulism were observed for a long time before van Ermengem (1897) isolated *Clostridium botulinum* and demonstrated that it produced a toxin. Most of the reported outbreaks of botulism have been caused by foods which were treated in the home by canning, pickling, or smoking. Among the foods most often incriminated in Europe in outbreaks of botulism have been sausages and underprocessed meats and in America home-canned vegetables, especially string beans, asparagus, spinach, peas, and corn. Canned fruits have been cited as the cause of an occasional outbreak. Investigations have revealed that the organism naturally occurs and is frequently found in surface layers of soil. It is logical to assume, therefore, that it may be present on vegetables and fruits which come in contact with soil, either during growth or preparation for the market.

Since 1925 no outbreaks of botulism have been caused by foods preserved in American canning factories. This is due largely to adoption of adequate processing times and temperatures and strict plant control. The advent of frozen foods, however, has revived interest in botulism. Since most frozen foods receive only very little heat treatment, it is conceivable that they might permit development of *Clostridium botulinum* were it present on the raw product. It is known that freezing does not completely sterilize a suspension of bacteria. Spores of *Clostridium botulinum* have been shown to be resistant to freezing, even at very low temperatures for relatively long periods. They may remain viable, therefore, and develop if a sufficient time interval elapses in thawed frozen foods along with other organisms which have survived freezing. Other organisms which may render the food unfit may also develop (Fellers, 1932). Wallace and Park (1933) and James (1933) observed viable spores in foods which had been frozen and stored at —16°C. (3.2°F.) for one year. Possibility of botulism occurring from thawed frozen foods was considered by Berry (1932) to be very slight. In

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a later paper Berry (1933) reported survival of lactic acid bacteria in frozen peas after two years. He considered these organisms to be of public health significance since they might exert an antagonistic influence on food-poisoning organisms such as *Clostridium botulinum*. It should also be noted that other organisms might favor development of *Clostridium botulinum*. One such instance was clearly indicated by Meyer and Gunnison (1929); Edmondson, Thom, and Giltner (1923); and Tanner and Wallace (1931). Organisms such as yeasts and molds may reduce acidity sufficiently, and aerobic bacteria may use up oxygen to such an extent that *Clostridium botulinum* may develop.

Experiments in our laboratories by Wallace and Park (1933) demonstrated that *Clostridium botulinum* may develop in inoculated, thawed, frozen foods and produce sufficient toxin to cause fatal results in laboratory animals. Results of the above investigation correlate well with data reported by Straka and James (1933). Brief review of their work reveals two facts which are significant to the frozen-food industry: (1) freezing does not destroy all spores of *Clostridium botulinum*; and (2) frozen foods which contain the organism or its spores may become toxic after thawing. One death has been reported owing to an imported canned fish product which had not been sterilized in the can and which was intended to be handled under refrigeration. The product had not been handled properly and was decomposed as evidenced by the swollen condition of the container (Anon. 1935). Prescott and Tanner (1938) stated that to their knowledge frozen foods have not caused botulism. There is little reason why they ever should if handled properly. The nearest approach is the death from botulism attributed to refrigerated, frozen, canned fish which had been mishandled.

Although this was not a frozen food, it may indicate possible consequences if frozen foods are improperly handled. Little hazard exists if fresh, clean, raw products are prepared and kept frozen until used by the consumer.

Another interesting question pertinent to this investigation is that of temperatures at which various strains of *Clostridium botulinum* may develop and cause food to be poisonous. Van Ermengem (1897) stated that the organism grew over a wide range of temperature, but optimum conditions were obtained between 20°C. (68°F.) and 30°C. (86°F.). Since then many investigations have been carried out to determine optimum temperature conditions for development of different strains of *Clostridium botulinum*. Temperature ranges between freezing and room temperature, from 0°C. (32°F.) to 20°C.

(68°F.), have been neglected until recently, although from the standpoint of food spoilage they are very important. Prescott and Geer (1936) arrived at conclusions which agree in general with those of Tanner and Oglesby (1936). The former men worked principally with but one strain of high virulence, while the latter investigators used 21 different strains. Tanner and Oglesby found that growth occurred and toxin was produced at 20°C.(68°F.) when detoxified spores were inoculated. At 15°C.(59°F.) germination and toxin production from heated spores occurred only rarely. Growth at 10°C.(50°F.) occurred only after 27 days and germination of spores had not taken place in 47 days at this temperature. Results obtained in this study seem to indicate that frozen foods stored at room temperature or under slight chilling conditions may become dangerous in a few days, but when kept below 10°C.(50°F.) will remain safe a considerable time. Usually at the higher temperatures of storage the foods show such evidences of spoilage that they would not ordinarily be prepared for the table.

The purpose of the present investigation is to seek further information on the ability of *Clostridium botulinum* spores to develop and produce toxin in frozen foods which have been thawed and held at various temperatures from 5°C.(41°F.) to 37°C.(98.6°F.) for as long as 14 days. Attempt was made to simulate conditions which would exist in actual usage.

EXPERIMENTAL PROCEDURE

Foods used in these experiments were divided into two groups: non-acid and acid foods. The non-acid group included peas, asparagus, green beans, spinach, sausage, and ground beef, which generally are less acid than pH 4.5. Black raspberries, cherries, and peaches constituted the acid group and had a pH more acid than 4.5. Twelve strains of *Clostridium botulinum*, four each of Types A, B, and C, were used. Identity of these strains was confirmed by typing with known antitoxic sera. Each of the strains of *Clostridium botulinum* used in this investigation had been isolated from food-poisoning outbreaks. All produced spores readily on casein digest medium and yielded potent toxins, absorption of which caused typical symptoms of botulism in guinea pigs.

Incubation at constant temperatures between 5°C.(41°F.) and 20°C.(68°F.) was accomplished by using electric refrigerators equipped with Freas heating and ventilating units.

Stock Spore Suspensions: In order to secure large yields of spores, casein digest medium, recommended by Sommer (1930), was prepared in large slants in six-ounce medicine bottles. Surfaces of

slants were swabbed heavily with cultures of the organisms grown in Difco egg-meat medium under vaseline seal. Slants were incubated at 37°C.(98.6°F.) 13 days in large jars rendered anaerobic with phosphorus, according to Varney's (1926) method. After removal from anaerobic jars, slants were stored at room temperature four days to enhance spore production. Growth from each slant was suspended in 20 cubic centimeters of M/15 disodium phosphate solution (previously adjusted to pH 7.0 and sterilized), pooled, and centrifuged 15 minutes at 1,200 r.p.m. to clear suspensions of debris and heavy clumps. After decanting, suspensions were shaken 10 minutes in sterile beaded bottles to break clumps of spores and thereby produce homogeneous suspensions. These stock spore suspensions contained approximately 120 to 140 million spores per cubic centimeter as determined by direct hemacytometer counts. Having been standardized, suspensions were stored at 5°C.(41°F.) until used.

Preparation of Food: Peas were shelled, asparagus and green beans were cut into one-inch pieces, sausages were cut into approximately 20-gram portions, cherries were pitted, peaches were sliced, and spinach leaves were used whole. Peas, asparagus, green beans, black raspberries, spinach, and cherries were washed in cold water to remove dirt and blanched three minutes in boiling water. Sausages and peaches were washed in cold water but were not blanched. All foods, with the exception of spinach, were weighed out, allowing approximately 20 grams to each container. Spinach leaves, because of their comparatively light weight, were weighed in 10-gram portions. Foods were placed directly into sterile, wide-mouth, glass bottles and the weight of container plus contents was recorded. About 50 cubic centimeters of 1:10 dilution of stock spore suspension, detoxified by heating at 100°C.(212°F.) for eight to ten minutes, were added, thoroughly mixed with the food, and the excess decanted. Weight of the container plus its contents was again recorded. Differences in weight in grams provided rough estimates of numbers of spores deposited on the food, generally between 20 and 60 million. Guinea pigs were fed one cubic centimeter of heated suspension to insure absence of potent toxin.

Freezing: Foods were frozen immediately after inoculation by placing in a previously cooled, well-insulated chamber. Use of solid carbon dioxide in the chamber provided temperatures of —14°C. (6.8°F.) to —20°C.(—4°F.). After two or three hours containers were removed from the chamber and placed at experimental temperatures in the controlled incubators to thaw and incubate. Uninoculated samples of fresh foods similarly prepared were also frozen and placed in incubators.

Testing Foods for Toxins: Presence of toxin in the thawed incubated food was determined by feeding one cubic centimeter of prepared food emulsion to a healthy, 250-gram guinea pig. Feedings were made after four days' incubation and again after fourteen days if the first feeding was negative. Emulsions were prepared by removing about five grams of food aseptically, adding approximately two cubic centimeters of sterile water, and emulsifying as well as possible with a sterile knife blade. In addition to feeding guinea pigs, one cubic centimeter was inoculated into egg-meat medium under vaseline seal. These tubes were incubated five days at 37°C.(98.6°F.) to test for development of viable spores present on the food. One cubic centimeter of culture was likewise fed to a guinea pig to determine toxicity. Guinea pigs were observed at intervals for 120 hours after experimental feedings.

DISCUSSION

Introduction of freezing as a method of food preservation presents new bacteriological problems. These are of a two-fold nature: those which relate to spoilage or deterioration and those which relate to possible health hazards. Frozen foods are perishable perhaps to a slightly greater extent than fresh foods, depending on the time required for freezing and histological changes produced. The fact that they have never caused illness is not proof that they are free from such possibility. Since they are perishable they must be handled properly to prevent development of microörganisms which may not be killed by freezing.

Results obtained in the present investigation (Table 1) agree with those formerly reported by Tanner and Oglesby. Growth occurred and toxin was produced in peas, asparagus, green beans, sausage, ground beef, and spinach within four days in 31 samples and within 14 days in 18 samples when the frozen foods were incubated at 20°C. (68°F.). Observations were in duplicate on all foods used with the exception of cherries, raspberries, and peaches. Tanner and Oglesby observed that when detoxified spores were inoculated into media, growth and toxin production rarely occurred at 15°C.(59°F.). Likewise, we found that toxin was produced in only nine samples when stored at 15°C.(59°F.) for four days; after incubation for 14 days, however, toxin was present in 10 samples of peas, asparagus, spinach, green beans, and two meat products. Toxin was found within four days in two samples of spinach and one sample of ground lean beef incubated at 10°C.(50°F.). Eight additional samples were toxic after 14 days' storage at this temperature: two samples of peas which had been inoculated with *Clostridium botulinum*, Type

TABLE 1

Development of Clostridium botulinum in Frozen Foods

PEAS

pH varied from 6.8 to 5.3 during incubation

Type of *Cl. botulinum*	Days incubated	Number guinea pigs fed	Deaths from peas stored at °C.					Toxicity of 5-day cultures of peas
			5	10	15	20	37	
A	4	20	0	0	1	3	3	No cultures made.
	14	13	0	0	0	0	1	
B	4	20	0	0	0	4	4	
	14	12	0	0	0	..	..	
C	4	20	0	0	0	2	3	
	14	15	0	2	3	0	0	

ASPARAGUS

pH varied from 6.9 to 5.2 during incubation

Type of *Cl. botulinum*	Days incubated	Number guinea pigs fed	Deaths from asparagus stored at °C.					Toxicity of 5-day cultures of asparagus
			5	10	15	20	37	
A	4	20	0	0	0	1	4	No cultures made.
	14	15	0	0	0	0	..	
B	4	20	0	0	0	0	4	
	14	16	0	2	0	4	..	
C	4	20	0	0	0	3	4	
	14	13	0	3	3	1	..	

GREEN BEANS

pH varied from 6.85 to 4.8 during incubation

Type of *Cl. botulinum*	Days incubated	Number guinea pigs fed	Deaths from green beans stored at °C.					Toxicity of 5-day cultures of green beans
			5	10	15	20	37	
								pct.
A	4	20	0	0	0	1	4	85
	14	15	0	0	1	3	..	
B	4	20	0	0	0	2	4	88
	14	14	0	0	0	0	..	
C	4	20	0	0	0	0	2	60
	14	16	0	0	0	4	0	

TABLE 1 *(Continued)*

SPINACH
pH varied from 6.8 to 5.3 during incubation

Type of *Cl. botulinum*	Days incubated	Number guinea pigs fed	Deaths from spinach stored at °C.					Toxicity of 5-day cultures of spinach
			5	10	15	20	37	
								pct.
A	4	20	0	0	1	0	4	100
	14	15	0	0	0	4	..	
B	4	20	0	2	1	2	3	100
	14	12	0	0	0	0	1	
C	4	20	0	0	1	1	4	100
	14	14	0	0	0	0	..	

SAUSAGE
pH varied from 6.6 to 7.8 during incubation

Type of *Cl. botulinum*	Days incubated	Number guinea pigs fed	Deaths from sausage stored at °C.					Toxicity of 5-day cultures of sausage
			5	10	15	20	37	
								pct.
A	4	20	0	0	2	2	4	100
	14	12	0	0	0	0	..	
B	4	20	0	0	0	1	4	100
	14	15	0	0	0	1	..	
C	4	20	0	0	0	0	2	95
	14	18	0	0	0	0	0	

HAMBURG STEAK
pH varied from 6.8 to 7.9 during incubation

Type of *Cl. botulinum*	Days incubated	Number guinea pigs fed	Deaths from hamburg steak stored at °C.					Toxicity of 5-day cultures of hamburg steak
			5	10	15	20	37	
								pct.
A	4	20	0	1	3	3	4	100
	14	10	0	1	1	1	..	
B	4	20	0	0	0	4	4	100
	14	12	0	0	2	..	..	
C	4	20	0	0	0	2	2	100
	14	16	0	0	0	0	1	

TABLE 1 *(Concluded)*

BLACK RASPBERRIES
pH 3.25

Type of *Cl. botulinum*	Days incubated	Number guinea pigs fed	Deaths from black raspberries stored at °C.					Toxicity of 5-day cultures of black raspberries
			5	10	15	20	37	
								pct.
A	4	20	0	0	3	0	0	100
	14	20	0	0	0	0	1	
B	4	20	0	0	0	0	0	100
	14	20	0	0	0	0	0	
C	4	20	0	0	0	0	0	100
	14	20	0	0	2	0	0	

CHERRIES
pH 3.75

Type of *Cl. botulinum*	Days incubated	Number guinea pigs fed	Deaths from cherries stored at °C.					Toxicity of 5-day cultures of cherries
			5	10	15	20	37	
								pct.
A	4	20	0	0	0	0	0	100
	14	20	0	0	0	2	1	
B	4	20	0	0	0	0	0	100
	14	20	0	0	0	0	2	
C	4	20	0	0	0	0	1	90
	14	20	0	0	0	0	0	

PEACHES
pH 3.8

Type of *Cl. botulinum*	Days incubated	Number guinea pigs fed	Deaths from peaches stored at °C.					Toxicity of 5-day cultures of peaches
			5	10	15	20	37	
								pct.
A	4	20	0	0	0	0	0	95
	14	20	0	0	0	0	0	
B	4	20	0	0	0	0	0	100
	14	20	0	0	0	0	0	
C	4	20	0	0	0	0	0	95
	14	20	0	0	0	0	0	

C; two samples of asparagus which had been inoculated with *Clostridium botulinum*, Type B; three samples of asparagus inoculated with *Clostridium Botulinum*, Type C; and one sample of ground beef which had been inoculated with *Clostridium botulinum*, Type A. A significant fact is that no deaths resulted in experimental animals which were fed foods incubated at 5°C.(41°F.). This observation bears out the contention that frozen foods are safe if properly refrigerated.

Foods held at 5°C.(41°F.) and 10°C.(50°F.) for 14 days showed no signs of spoilage or decomposition. Original color was retained and pH remained unchanged. Even those which were toxic showed no visible signs of spoilage after 14 days' incubation at 10°C.(50°F.). In general, peas, asparagus, spinach, and green beans changed from pH 6.8-6.9 to 4.8-5.2 during incubation at 15, 20, and 37°C.(59, 68, and 98.6°F.). Ground beef and sausage became more alkaline, changing from pH 6.6-6.8 to 7.8-7.9. Particularly with those samples held at 20 and 37°C.(68 and 98.6°F.) decomposition was evidenced by strong putrefactive odors and loss of color, as would be expected.

In 1922, Cutter reported a death from food poisoning following ingestion of a tomato-onion-chili sauce. Meyer isolated *Clostridium botulinum* from the acid sauce. This was the first reported case of botulism caused by an acid product. Meyer and Gunnison (1929) studied a sample of home-canned Bartlett pears, pH 3.86, which caused two deaths. They isolated *Clostridium parabotulinum A* from the pear syrup and also a yeast and a member of the Lactobacillus group. Meyer believed that *Clostridium botulinum* may elaborate toxin in pears, irrespective of acidity, providing bacteria or yeasts of certain types are present. Observations such as this have stimulated research concerning the limiting hydrogen-ion concentration for toxin production and also concerning effects of other organisms growing concomitantly with *Clostridium botulinum*.

Since frozen foods are probably never sterile, organisms which favor development of *Clostridium botulinum* in acid media may be present. We have studied toxin production in black raspberries (pH 3.25), cherries (pH 3.75), and peaches (pH 3.8). Presence of toxin in fruits incubated at 5 and 10°C.(41 and 50°F.) was not observed after 14 days. Five samples of black raspberries incubated at 15°C. (59°F.) caused botulism in experimental animals; in three of these, toxin was detected in four days. A mold, tentatively identified as *Penicillium*, was growing in the food and pH of the juice had been altered to 5.4. In two samples showing toxin after 14 days' incuba-

tion, a yeast identified as a *Mycoderma* species had changed the pH to 4.9.

Two portions of cherries incubated 14 days at 20°C. (68°F.) were toxic as were four samples incubated at 37°C.(98.6°F.). In all six samples we observed profuse growth of a yeast possessing characteristics of *Mycoderma*. The pH in these toxic samples had changed from 3.75 to 5.1-5.3. Cultures of uninoculated peaches and cherries were not toxic, whereas cultures of inoculated black raspberries were toxic in every case.

Further studies concerning development of *Clostridium botulinum* in acid foods are being continued in our laboratories. It is hoped to include also comprehensive study of relationships between *Clostridium botulinum* and other microörganisms commonly found in frozen foods.

Although the work is not directly pertinent to this investigation, Jones and Lochhead's (1939) results showing that enterotoxin-forming micrococci may be present in frozen foods is interesting. None of the strains produced enterotoxin in defrosted corn at 4°C. (39.2°F.) to 10°C.(50°F.).

SUMMARY

Development of 12 strains of *Clostridium botulinum* has been studied in nine frozen foods of different acidity stored at 5°C. (41°F.), 10°C.(50°F.), 15°C.(59°F.), 20°C.(68°F.), and 37°C. (98.6°F.).

Production of toxin occurred consistently in foods inoculated and stored at 37°C.(98.6°F.). These foods were included in the study as controls.

In general, detoxified spores of *Clostridium botulinum* may germinate and produce toxin in foods more alkaline than pH 4.5 stored at 20°C.(68°F.) and 15°C.(59°F.). Occasionally toxin production occurred in non-acid foods at 10°C.(50°F.).

Inoculated frozen foods, acid and non-acid, stored at 5°C.(41°F.) were not toxic after 14 days.

Certain samples of acid foods, pH less than 4.5, were toxic. Species of *Penicillium* or *Mycoderma* were found in these samples and the pH was altered so the food was more alkaline than pH 4.5.

Our data indicate that *Clostridium botulinum* may sometimes develop and produce toxin in frozen foods which have been allowed to thaw and have been stored at temperatures 10°C.(50°F.) and above. However, if properly handled and kept frozen until used, frozen foods are probably as safe and satisfactory as fresh foods.

In all of these experiments great numbers of *Clostridium botulinum* cells were used. Much heavier inoculation was used than would probably ever be found in the regular product.

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RELATION OF MIXING METHODS AND A BALANCED FORMULA TO QUALITY AND ECONOMY IN HIGH-SUGAR-RATIO CAKES[1]

W. E. PYKE AND GESTUR JOHNSON
Home Economics Section, Colorado Agricultural Experiment Station, Fort Collins, Colorado

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PART I

The method commonly used in the mixing of "butter-type" cake batters is to first "cream" the sugar and shortening until well blended and as light as possible. Gradually, then, the beaten or unbeaten eggs are incorporated into the creamed mass. Finally, the flour sifted with the baking powder is added gradually and alternately with the milk.

The "single-stage" method of mixing is widely used by chemists in cake-flour baking tests. Bailey and LeClerc (1935) have demonstrated that excellent cakes of the heavier type (butter cakes) can be made by this process of mixing. This method consists of adding all of the ingredients except the leavening to the mixing bowl and mixing at low speed from one-half to one minute. The speed is changed to medium and mixing is continued for 10 minutes. Baking powder (or the acid constituent, if soda and acid are used) is then added and mixing continued from one to two minutes. According to Bailey and LeClerc, "The 'single-stage' method of mixing saves time and is subject to fewer variations than the 'creaming' method. Its use makes for uniformity in production."

EXPERIMENTAL PROCEDURE

The method of mixing is dependent to a considerable extent upon the nature and properties of the ingredients used. In this study only high-grade commercial cake flour, that had not been diluted by a stream of finely ground hard wheat flour in the blending at the mill, was used. This type of flour had proved to be the most economical and invariably yielded cakes of higher quality. Its moisture content varied between 11 and 12 per cent. The eggs were fresh and of high quality. Their quality corresponded to egg scores of less than three on the Van Waggenen-Wilgus Score Card (1935). Commercial hydrogenated shortening was used because it was found that butter would not tolerate the use of the quantity of liquid in the batter that this study demanded. Shortenings containing added emulsify-

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ing agents were compared with ordinary hydrogenated shortening containing no added emulsifying agents. The studies in this report were carried out at an altitude of 5,000 feet. In Part II of this paper they will be adapted to all practical altitudes.

Only the whole-egg cake was involved in this study. Experiments began with the basic test formula for the whole-egg, butter-type cake developed for use at 5,000 feet altitude by the Colorado Experiment Station. To get a satisfactory idea of the adaptability of mixing methods to the various types of cake batters now in use it seemed necessary to consider the rebalancing of this basic formula to include an indefinite series of cake formulas varying in their sugar, egg, and liquid contents. It also seemed desirable to learn whether special hydrogenated shortening containing an emulsifying agent contributed properties to the mix which rendered its use advisable, or whether satisfactory techniques could be developed whereby ordinary hydrogenated shortening could be employed. The basic formula (5,000 feet altitude) to be adapted to these different conditions was as follows:

Flour	300 grams	Water	180 c.c.
Sugar	275 grams	Salt	4.5 grams
Shortening (Commercial hydrogenated)	130 grams	Soda	2.3 grams
Milk (dry skim)	27 grams	Cream of tartar	4.6 grams
Egg white	90 grams	Vanilla	5 c.c.
Egg Yolk	54 grams		

In the use of this formula 280 grams of batter were weighed into each of three 8 x 4 x 2½-inch pans and baked at 175-177°C.(347-350.6°F.) for 40 minutes.

Findings of this laboratory indicate that with batters whose water contents vary between 90 and 100 per cent of the weight of the flour, the specific gravity is more easily controlled by the single-stage method of mixing. Microscopic examination of batters mixed by the creaming and single-stage methods indicates that there is more fat accumulated at the air-water interface when the mixing has been by the single-stage method.

When batters with water content greater than 110 per cent on the flour basis are mixed by either of these methods, a distinct tendency to "curdle" is noted where ordinary hydrogenated shortening is used. If the liquid content is high enough, the batter will curdle so badly and become so unstable that separation into two distinct layers may occur during the baking process (cake No. 606, Fig. 1).

A curdled batter has a much higher specific gravity and lower viscosity than a stable one. Such a batter yields a cake of lower volume, coarser grain, and a somewhat harsher, rubbery texture, which is not of the desirable velvety type. A smooth and a curdled batter are compared (Fig. 2).

Lowe (1932) has pointed out that the fat distribution in the cake batter is a very important contributing factor in the development of a velvety texture in the finished product. To yield a velvety texture the fat must not be distributed in thick layers, nor should an

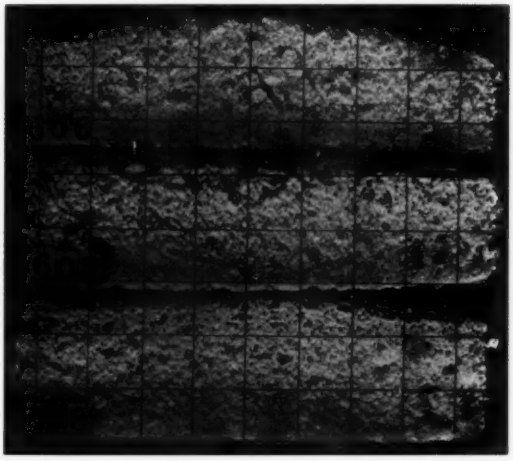


FIG. 1. Comparison of cakes mixed by three different methods.

606. Cake mixed by single-stage method. Note separation and heavy layer at bottom.

607. Cake mixed by the creaming method. Cake is coarse, of poorer volume, and tougher than 608.

608. Cake mixed by the modified single-stage method. All three cakes contained 133 per cent sugar on the flour basis and were five-egg cakes.

oil-in-water emulsion be produced. Rather, an intermediate mixture should be formed where the fat is present in very thin films at the air-liquid interface and upon the surfaces of the ingredient particles of the batter.

By a slight modification of the single-stage method of mixing, excellent results have been obtained in this laboratory using *ordinary* hydrogenated shortening in the preparation of batters rich in sugar, liquid, and eggs. This procedure consists essentially in holding back a portion of the liquid (water or milk) until a stable, smooth batter has been formed. The remaining liquid is then added slowly, fol-

lowed by baking powder or the acid ingredient of baking powder if sodium bicarbonate and acid are used. A comparison is made of batters high in liquids containing the same ingredients (Fig. 2); the curdled one was mixed by the single-stage method of mixing, the smooth one by the modification of the single-stage method just described.

In the use of this method enough liquid is held back so that the initial water content of the batter for the first part of the mixing period is between 90 and 100 per cent of the weight of the flour used. This first part of the mixing period is continued for eight minutes, when the batter will have reached its maximum specific volume. The change in specific volume of the batter with time is indicated (Fig. 3). At the point numbered 1, it has ceased to become lighter

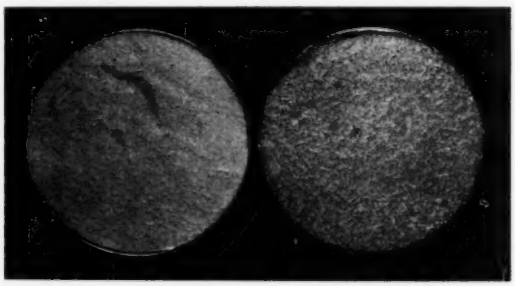


FIG. 2. Effect of method of mixing on smoothness of batter.

Smooth batter at left by modified single-stage method of mixing, ordinary hydrogenated shortening used in batter, with 126 per cent sugar on the flour basis. Curdled batter at right obtained from same ingredients, using the single-stage method of mixing.

with continued mixing. The point A indicates the corresponding viscosity in degrees MacMichael. For the viscosity determinations, 22 revolutions per minute with a No. 26 wire and small plunger and cup were used. The sample was 25 c.c. of batter at 22-23°C. (71.6-73.4°F.).

After the maximum volume had been obtained at Point 1, 35 grams of sugar dissolved in 35 grams of water were added and mixing was continued two minutes. The specific volume had dropped slightly, but addition of 10 grams of shortening and beating for two minutes raised it to Point 2. The corresponding changes in viscosity are represented by Points B and C. As this process is repeated, specific volume changes to Points 3 and 4 are obtained with viscosity changes represented by D, E, F. and G. The changes represented by Points 5 and H were obtained by the use of one-half an additional

increment of sugar and water. If the various increments of sugar and water are added without the addition of fat, a very rapid drop of viscosity occurs and the specific volume of the batter is in no wise maintained. If the amount of fat represented by Point 4 is added initially along with the total sugar but only sufficient water is present to equal approximately 100 per cent on the flour basis, mixing will produce the specific volume represented by Point 4. Upon addition of the remainder of the water required to properly balance the formula, the specific volume is maintained, even though a considerable drop in viscosity results.

The superior results obtained by this method of mixing are attributed to the fact that the initial smooth batter first built up is

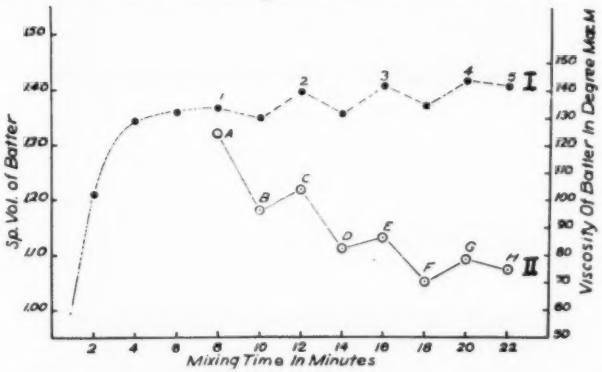


FIG. 3. Effect of mixing time and ingredient balance upon batter properties.

I. Specific volume of batter.
II. Viscosity of batter.

sufficiently stable to tolerate the remaining liquid without excessive curdling. Cakes produced from such a batter have a better texture and are generally more desirable than those obtained by raising with increased baking powder an unduly heavy batter of low viscosity (Fig. 11).

For only a relatively short time, shortening manufacturers have had on the market in addition to their regular hydrogenated shortening, a special product containing a small amount of emulsifying agent. This shortening is recommended especially for "high-sugar-ratio" cakes. Regardless of the mixing method used, this specially prepared fat gives a smoother though heavier batter than ordinary shortening. The single-stage method of mixing gives excellent results when this special shortening is used in batters high in liquid and sugar. Some of these special products give better volume when the

modified single-stage method just described is used; others give better results using the single-stage method. None of the special shortenings tried gave as good volume by the creaming method as by

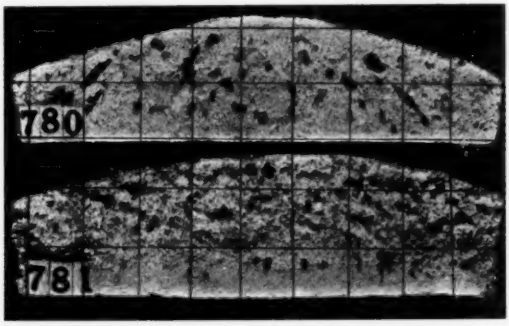
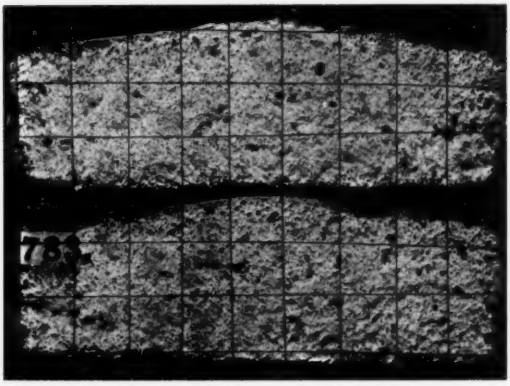


FIG. 11. Cakes from suitable and unsuitable shortening.

780. This cake was made by the modified single-stage method of mixing. The fat used for shortening had a very low water tolerance. The batter was almost as fluid as water. Its specific volume was below one.

781. A cake from the same batter as used for cake No. 780. The amount of leavening has been doubled. Improvement in volume and texture are noted but the product is still very unsatisfactory. This represents about the maximum improvement possible with increased leavening upon an unstable batter.



783. Samples from a cake made by the same method of mixing as No. 780. Ordinary hydrogenated commercial cake shortening was used as the fat. Desirable texture and economical volume have been obtained.

(The formula used is given at the bottom of Table 6.)

either the single-stage method or the modified single-stage method. Cake volumes obtained by the modified single-stage method when ordinary hydrogenated shortening was used were consistently some-

what larger than those obtained when these special cake shortenings were used. The texture and keeping qualities of these cakes were fully as good as, if not superior to, those resulting from the special shortenings.

In agreement with the observations of Fisher (1936), consistently better results were obtained through the use of the whip for mixing cake batters. This applies to all steps except the blending of sugar with the fat in the creaming method, where the use of the paddle or beater is desirable. When the paddle is used in the above-described

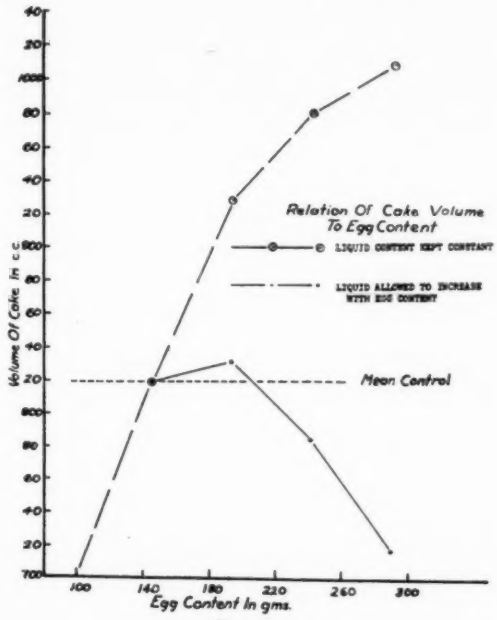


FIG. 4.

modified single-stage method, it is practically impossible to prevent a decided increase in specific gravity when the supplementary liquid is added toward the end of the mixing process, even when low speed is used. With the whip, lighter batters are obtained and the supplementary liquid may be added at second speed, which is used in the eight-minute batter-building period.

DISCUSSION OF RESULTS

In the series of experiments necessary to balance properly the basic whole-egg cake formula given previously, the relationship of cake volume to the egg content of the batter had been obtained. These

determinations included variation of egg content, keeping the liquid content of the batter constant, and variation of the egg content, allowing the liquid content of the batter to vary by the amount of liquid present in the fresh egg. These results are given (Tables 1 and 2) and are indicated graphically (Fig. 4).

TABLE 1

Effect of Increasing Fresh-Egg Content on Volume of Cake at 5,000 Feet Altitude

(Liquid content kept constant)

Number of eggs	White	Yolk	Mean volume	Mean variance	Number of cakes
	gm.	*gm.*	*c.c.*		
2	60	36	646.2	169.37	6
3	90	54	817.5	316.26 [1]	20
4	120	72	928.1	221.37	9
5	150	90	981.6	172.96	9
6	180	108	1,008.1	80.53	6

(Grand mean volume = 869.2)

Source of variation	Sum of squares	Degrees of freedom	Mean variance
Between groups	612,517.85	4	153,129.46 [2]
Within groups	9,990.63	45	220.14

[1] Different cake flours and both summer and winter types of shortening were used in three-egg cake, yielding a much higher variance. [2] Highly significant.

TABLE 2

Effect of Increasing Fresh-Egg Content on Volume of Cake at 5,000 Feet Altitude

(Liquid varies with added egg)

Number of eggs	White	Yolk	Mean volume	Mean variance	Number of cakes
	gm.	*gm.*	*c.c.*		
3	90	54	817.5	316.26	20
4	120	72	831.4	269.28	9
5	150	90	784.8	818.20	9
6	180	108	718.1	521.62	9

(Grand mean volume = 794.9)

Source of variation	Sum of squares	Degrees of freedom	Mean variance
Between groups	76,207.70	3	25,402.57 [1]
Within groups	18,881.78	43	439.11

[1] Highly significant.

The influence of egg content upon the tensile strength of these cakes at 5,000 feet is given (Tables 3 and 4).

It is clear that an increase in the egg-solids content of the batter produces an increase in cake volume (Fig. 4). The volume deter-

minations were by the seed displacement method. If allowance is not made for the water added as a portion of the egg, however, the change produced quickly passes through a maximum and then the volume decreases rapidly. Tensile-strength data show that increase in egg

TABLE 3

Effect of Increasing Fresh-Egg Content on Tensile Strength of a Whole-Egg Cake Containing Shortening at 5,000 Feet Altitude

(Liquid content kept constant)

Number of eggs	White	Yolk	Mean tensile strength	Mean variance	Number of cakes
	gm.	*gm.*	*gm./cm.²*		
2	60	36	10.5	3.13	5
3	90	54	11.2	0.70	20
4	120	72	13.3	1.12	9
5	150	90	15.8	0.14	9
6	180	108	17.8	1.21	6

(Grand mean tensile strength = 13.5)

Source of variation	Sum of squares	Degrees of freedom	Mean variance
Between groups	325.60	4	81.40 [1]
Within groups	41.95	44	0.95

[1] Highly significant.

TABLE 4

Effect of Increasing Fresh-Egg Content on Tensile Strength of a Whole-Egg Cake Containing Shortening at 5,000 Feet Altitude

(Liquid content varies with liquid content of egg)

Number of eggs	White	Yolk	Mean tensile strength	Mean variance	Number of cakes
	gm.	*gm.*	*gm./cm.²*		
3	90	54	11.2	0.70	20
4	120	72	15.2	0.54	9
5	150	90	18.4	3.03	9
6	180	108	21.0	2.35	9

(Grand mean tensile strength = 15.2)

Source of variation	Sum of squares	Degrees of freedom	Mean variance
Between groups	714.92	3	238.31 [1]
Within groups	60.72	43	1.41

[1] Highly significant.

content produces a tougher cake. When the influences of the variation of sugar content and of shortening content upon the volume of the cake are followed, similar bell-shaped curves are obtained. The means obtained from a mass of comparable data have been plotted

(Fig. 5). Since there is no doubt regarding the significance of these values, the statistical treatment has been omitted. The standard error of a point is ± 2.37 c.c. for sugar and ± 2.74 c.c. for shortening. It is evident that when any one of these constituents is varied separately the volume of the cake passes through a well-defined maximum. The area between the upper and lower branches of the curve of Fig. 4 shows the influence that approximately 35-gram increments of water have upon cake volume.

When the proper balance between these ingredients is maintained as they are varied (the amount of flour still remaining constant at

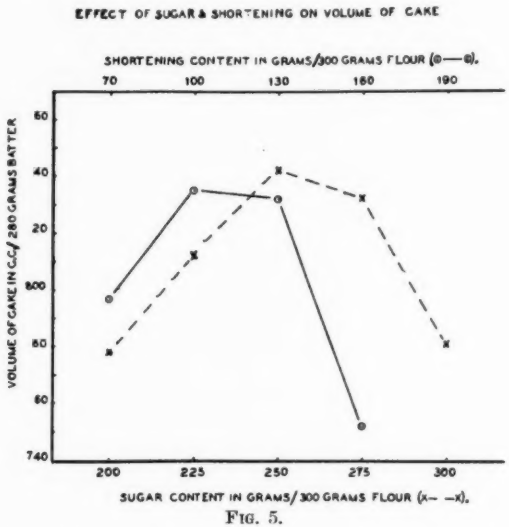


FIG. 5.

300 grams), the volume may be maintained practically unchanged also. The effect of water and sugar variation when they are added in equivalent amounts is evident (Fig. 6, Curve III); the 35-gram increment was chosen because this represents the water content of the average egg. In Curve II may be noted the improved effect of adding the 35 grams of water in the form of an egg. The difference in batter composition on Curve II as compared with Curve III is simply the solids of one egg for each increment of sugar. Egg solids have been able to maintain the volume for an added 70 grams each of sugar and water, the water being a part of the added whole egg. Curve I shows that a 10-gram increment of shortening added with the sugar, liquid, and egg-solid increase not only gives a small but significant increase in volume but also serves to maintain that

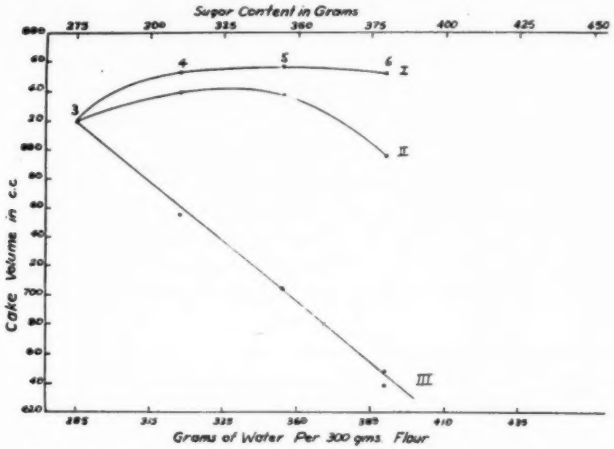


FIG. 6. Effect of balancing of ingredients upon cake volume.

I. Variation of balanced ratios of sugar, water, egg solids, and shortening. Shortening variation = 10 grams for each increase of one egg. Numbers along curve indicate the egg variation in whole eggs.

II. Variation of sugar, water, and egg solids in balanced ratios. Shortening held constant.

III. Variation of sugar and water in proper ratio. Egg solids and shortening are held constant.

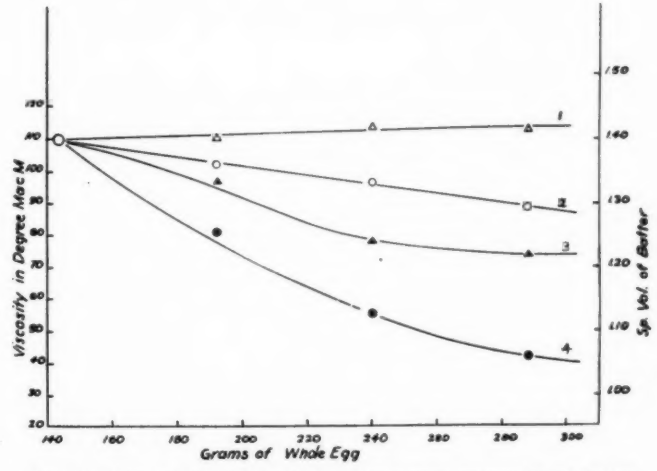


FIG. 7. Effect of balancing of ingredients upon properties of batter.

1. Specific volume of batters of balanced formulas of Curve I, Fig. 6.
2. Specific volume of batters of Curve II, Fig. 6.
3. Viscosity of batters of Curve I, Fig. 6.
4. Viscosity of batters of Curve II, Fig. 6.

volume into the realm of sweeter cakes. The number of eggs used per 300 grams of flour is indicated for Curves I and II by the numbers along Curve I; each point on Fig. 6 represents the means of six cakes. The standard error of a point is ± 1.57 c.c.

The variation of two important physical properties of the batters represented in Curves I and II of Fig. 6 is shown (Fig. 7). The triangles indicate the trends of the mean specific volume (Curve 1) and viscosity (Curve 3) for the batters of the balanced formulas represented in Curve I, Fig. 6, where shortening was increased along with sugar, water, and egg solids. The circles (Curves 2 and 4) follow the corresponding changes (Curve II, Fig. 6), where the amount

TABLE 5

Balanced Whole-Egg Cakes With Sugar Content Optional[1]

Per cent of sugar on flour basis	92	103	115	127	138	144
Ingredients:[2]						
Cake flour	300	300	300	300	300	300
Sugar	275	310	345	380	415	432
Dry milk (skim)	27	27	27	27	27	27
Egg white	90	120	150	180	180	180
Egg yolk	54	72	90	108	108	108
Water	180	180	180	180	215	232
Shortening (hydrogenated)	130	140	150	160	160	160
Salt[3]	4.5	4.5	4.5	5	5	5
Baking powder	8.6	8.6	8.6	8.6	8.6	8.6
Vanilla	5	5	5	5	5	5

[1] These formulas are balanced for use at 5,000 feet. [2] All measurements are in grams weight. [3] Little difference in salinity can be detected between cakes containing 4.5 or 5 grams of salt. Arbitrarily 5 grams of salt have been used in the larger batches. Salinity has been kept sufficiently low to permit the use of a butter cream topping if desired. If no frosting is to be used, the cakes will tolerate one gram more salt without the introduction of too great salinity in the flavor. Variation of the salt within the limits suggested will have no appreciable effect upon the cake texture.

of shortening used remained constant. By a proper balance of these four ingredients of the batter, it has been possible to maintain a desirable specific volume of the batter as the sugar and water content increases, even though a considerable decrease in viscosity occurs. With constant shortening content, the batter thins out much more rapidly as sugar and water are increased.

The series of formulas shown (Table 5) represent a variation in sugar content on the flour basis from 92 to 144 per cent. These formulas are applicable at 5,000 feet altitude. This represents a change in richness from an average cake to a rich high-sugar-ratio cake. These formulas were obtained by suitable modification of the basic formula at the left in the table. The modification of the single-stage

method described was found to give excellent results with the whole range of formulas indicated when ordinary hydrogenated shortening was used.

The mean values of several properties of cakes and the batters from which they are made are compared (Table 6). These values are representative of what are obtained when ordinary hydrogenated shortening is employed with the different methods of mixing used. Special shortenings Nos. 1 and 2 represent the group of commercial hydrogenated shortenings containing added emulsifying agents. These two shortenings are the most widely used by commercial bakers in this locality. They gave the best volume response of the group of special shortenings. Cakes from these shortenings were very fine-grained, tender, and of high eating quality. They were perhaps slightly finer grained than the cakes made from ordinary hydrogenated shortening by the modified single-stage method of mixing but if so, the difference was certainly slight.

An important fact regarding a properly balanced cake mix is brought out (Tables 5 and 6); the total water content of the batter should increase as rapidly as the sugar content or the cake will not bake properly. This is due to the fact that neither egg proteins (see Barmore, 1936) nor starch (see Woodruff, Sybil, and Nicoli, Laura, 1931), will set to a gel at the temperature of a baking cake if the sugar-water ratio exceeds a certain value. This value varies with the variation of the maximum internal temperature attained within the baking cake. The relation of this maximum internal temperature to the boiling point of the internal solution will be discussed in another paper. In connection with the present discussion and the series of formulas used in the baking tests, this requirement had to be fulfilled. From the standpoint of satisfactory coagulation and gelation it makes no difference whether this water is added as pure water or as a constituent of some other ingredient (the egg or milk used). When the quality and economical volume of the cake produced is taken into account, however, it may be very desirable to add this required water as added egg or milk; to emphasize and clarify this point, Fig. 8 is presented. It will be noted that the most economical egg content for the 127 per cent sugar cake is four or five eggs with little difference in the cost if the five-egg formula is chosen. In other words the five-egg formula has given enough volume increase to make up for the difference in the cost of the added egg and shortening increment. This increase in volume has been sufficient also to offset the toughening effect of increased egg content. As a result the tensile strength of the cakes remains in the desirable range. It was this formula which was used for the comparison of shortenings shown (Table 6). The values used in computing the

TABLE 6

A Comparison of Shortening Behavior

Type of shortening	Method of mixing	Type of beater	Mean specific volume of batter	Mean volume of cakes	Mean specific volume of cakes	Mean tensile strength of cakes	Remarks
				ml.		*gm./cm²*	
Ordinary hydrogenated.....	Modified single-stage	Wire whip	1.39	823	3.56	10.4	.....
Ordinary hydrogenated.....	Modified single-stage	Paddle	1.25	708	3.06	15.4	.....
Ordinary hydrogenated.....	(Modified) creaming	Paddle	1.24	760	3.26	15.1	.....
Special No. 1.....	Modified single-stage	Wire whip	1.26	762	3.31	9.9	Crumbly cake
Special No. 1.....	Modified single-stage	Paddle	1.23	741	3.28	10.4	Crumbly cake
Special No. 1.....	Single-stage	Wire whip	1.35	791	3.50	9.6	.....
Special No. 2.....	Single-stage	Wire whip	1.24	768	3.34	10.4	.....
Special No. 2.....	Modified single-stage	Wire whip	1.31	808	3.53	10.2	.....
Butter (for comparison).....	Modified single-stage	Wire whip	0.967	598	2.58	.....	(Cake separated)
Lard (for comparison).....	Modified single-stage	Wire whip	0.865	530	2.15	.....	(Cake separated)

Formula used (grams wt.)

Cake flour	400	Salt	5.0
Whole eggs	240	Soda	2.3
Sugar	380	Cream of tartar	4.6
Shortening	150	Water	215.0
Dry milk (skim)	27	Vanilla	5.0

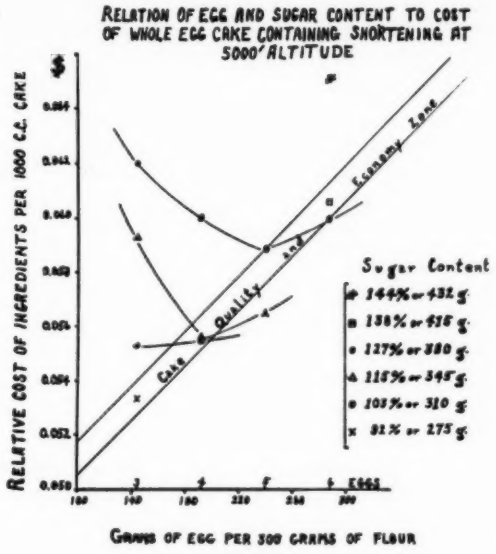

RELATION OF EGG AND SUGAR CONTENT TO COST OF WHOLE EGG CAKE CONTAINING SHORTENING AT 5000' ALTITUDE
RELATIVE COST OF INGREDIENTS PER 1000 C.C. CAKE
$
Economy Zone
Quality and Cake
Sugar Content
144% or 432 g.
138% or 416 g.
127% or 380 g.
115% or 345 g.
103% or 310 g.
92% or 275 g.
GRAMS OF EGG PER 300 GRAMS OF FLOUR

FIG. 8.

Low-cost, three-egg, high-quality cake contains 92 per cent sugar.
Low-cost, four-egg, high quality cake contains 103 per cent sugar.
Low-cost, five-egg, high-quality cake contains 115 per cent sugar.
Low-cost, six-egg, high-quality cake contains 127-138 per cent sugar.
Cakes having above 138 per cent sugar should contain at least six eggs to keep quality up and cost per unit down at an altitude of 5,000 feet.

relative cost of ingredients of the cakes shown (Fig. 8) are arbitrary. These values follow:

300	grams cake flour	$0.0330
27	grams dry skim milk	0.0059
5	grams salt	0.0005
2.3	grams soda	0.0003
4.6	grams cream of tartar	0.0031
5	grams flavoring	0.0053
30	grams egg white	
18	grams egg yolk (1 egg)	0.0200
275	grams sugar	0.0303
130	grams shortening	0.0345

SUMMARY

Difficulties arising from the use of ordinary commercial hydrogenated shortening in the preparation of whole-egg, butter-type cakes high in sugar, liquid, and eggs have been overcome by a suitable modification of the single-stage method of mixing.

Cake batters prepared by this modified single-stage method of mixing, when ordinary hydrogenated shortening was used, had a greater specific volume than batters prepared from special hydrogenated shortenings containing added emulsifying agents, regardless of the mixing method used with these special shortenings.

Cakes baked from batters prepared by the modified single-stage method of mixing with ordinary hydrogenated shortening had significantly greater volumes than those produced through the use of shortenings containing emulsifiers. These cakes were of fully as high quality as those prepared from special shortenings.

Batters from special shortenings containing emulsifiers were invariably smoother and heavier than batters from ordinary hydrogenated shortening. Cakes of smaller volume resulted from these batters.

Any attempt to raise unduly heavy batters by increased baking powder content produced a less desirable structure and texture than when a light batter with a desirable specific volume was baked with the proper amount of baking powder. In fact these heavy batters tended to give quite coarse products whenever it was attempted to increase their volume by an increase in the leavening.

A basic, whole-egg cake formula lean in sugar, eggs, and liquid has been rebalanced stepwise with added increments of sugar, egg-solids, shortening, and liquid. In this way a desirable specific volume may be maintained in the finished batter and the cake produced. When any of these ingredients are increased, the others should be suitably increased simultaneously if the high quality of the cake is to be maintained. When a proper balance of ingredients is maintained as the richness of the cake increases, cakes of comparable volume, texture, and quality but of considerable difference in nutritional value may be produced. Cakes of optional sweetness are thus possible.

The water requirement of a cake batter is established to a large extent by its sugar content. Water cannot be decreased below a certain definite minimum for any formula and set of baking conditions without failure of the egg proteins to coagulate and the starch to gel. The specific effect of sucrose upon the rate of set of these constituents with variations in temperature is responsible for this behavior.

Quality and economy in cakes are related to the proper balance of the cake formula used. If high-sugar-ratio cakes are desired, the formula used should be rebalanced for each increase in sugar content if high quality and sound economy are to be maintained.

The type of mixing equipment chosen and the method of mixing used are as important in maintaining high quality and sound econ-

omy in cakes as the use of a properly balanced formula. An understanding of the relation of mixing methods to the properties of available ingredients permits the selection of those ingredients which will yield high quality and at the same time keep costs at a low level.

PART II

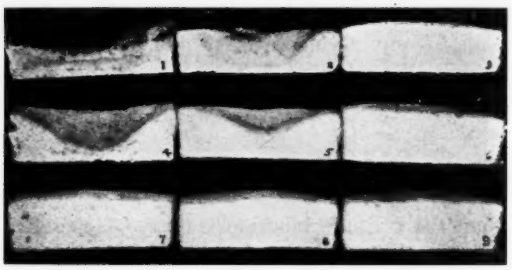
REBALANCING THE CAKE FORMULA FOR DIFFERENT ALTITUDES

For a period of years the altitude laboratory of the Home Economics Section of the Colorado Experiment Station has continued a study of the effect of altitude or air pressure upon the events of the baking process. Certain features of this work are of considerable general interest and importance. In Part I the relation between methods of mixing, the balanced formula, quality, and economy of high-sugar-ratio cakes at a particular altitude (5,000 feet) was discussed. The effect of altitude upon the baking cake will now be considered. The balanced formulas presented in Part I will be extended so that cakes of comparable quality and economy but with optional sweetness may be prepared at any practical altitude.

Only a whole-egg cake containing shortening will be considered. The characteristics of the whole-egg cake recommend it above all other cakes containing shortening from the standpoint of quality and economy. The whole-egg cake forms the foundation mix for many popular specialties. The features involved in adjusting the formulas of foundation butter cakes to meet the conditions imposed by a change in the altitude at which the formula is baked are not wholly unlike adjustments that are necessary to change a foundation formula into an attractive specialty. In each of these cases a disturbance in the balance of the formula occurs. The principles involved in rebalancing the formula after the balance has been disturbed are the same. The manner in which the disturbance is brought about is immaterial.

As in Part I of this study only high-quality, soft-wheat cake flour was used. The shortening was ordinary commercial hydrogenated shortening containing no added emulsifying agent. Fresh eggs of the high quality described before were used. The modified single-stage method of mixing with the wire whip on the Hobart C-10 mixer was used.

When the pressure against which a cake batter expands is decreased, the volume the leavening gases will attain in their expansion during baking will be proportionally increased. Naturally any determined quantity of batter will permit only a certain degree of expansion "before the bubble bursts." As this pressure is decreased,



Altitude

⇄ 10,000 feet

⇄ 5,000 feet

⇄ Sea level

↑↓ ↑↓ ↑↓

Sea level { Sea level with baking powder correction for altitude { Correctly balanced formulas for altitude .....⇄ FORMULA USED

	Ingredients	Weight in grams for altitude indicated		
		Sea level	5,000 ft.	10,000 ft.
FORMULAS:	Cake flour	300	300	300
	Sugar	350	350	350
	Shortening	130	140	150
	Egg white	90	120	150
	Egg yolk	54	72	90
	Dry milk (skim)	27	27	27
	Water	201	220	220
	Salt	4.5	4.5	4.5
	Baking powder	12.9	8.6	1.08
	Vanilla	5	5	5

FIG. 12. Corrected and uncorrected whole-egg, butter-type cake formulas baked at various altitudes.

therefore, the volume which the leavening gases may produce during the process should be limited correspondingly (Fig. 12). In the control of the volume attained by the leavening gases in the oven, many diverse factors are involved. Notably, these factors are the quantity and nature of baking powder, the relative amounts and quality of the various ingredients, the length and character of the mixing period, and the temperature rise of the batter. Perhaps the correction of the amount of baking powder or other chemical leavening agent to be used is the most generally recognized factor to be controlled as the site of baking changes from one altitude to another. However, in the complete absence of any chemical leavening agent, it is mechanically possible to incorporate enough air into a batter in the mixing process so that the batter will be overleavened. Techniques used in mixing and the type of equipment used, therefore, are important.

That liquids boil at lower and lower temperatures as the pressure upon them is decreased is an established fact. That the internal

temperature attained by a cake during baking is limited by the boiling point of water under the conditions of the experiment is shown (Fig. 9). These values for three different altitudes were obtained by the use of thermocouples by a procedure similar to the one described by Barmore (1936). The basic formula for the whole-egg, butter-type cake was used in this series of experiments. The results are in agreement with the determinations of Barmore upon angel food cake.

The cake batter is a water mixture. The temperature at which the baking changes are effected on the various ingredients may exceed

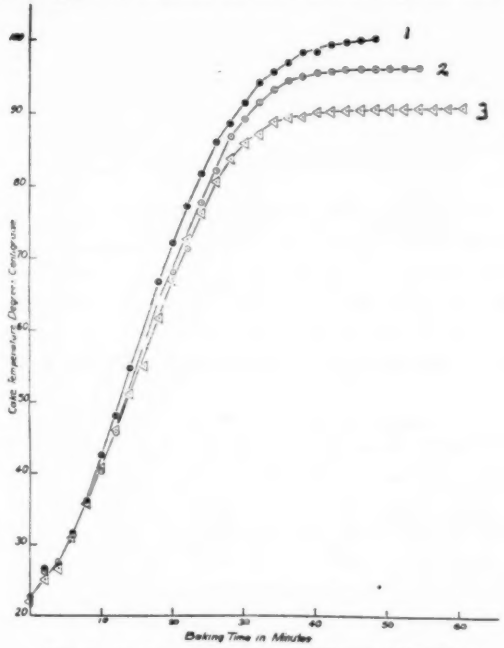


FIG. 9. Heat penetration for different altitudes.
(Whole-egg, butter-type cake)

1. Heat penetration curve for sea level.
2. Heat penetration curve for 5,000-foot altitude.
3. Heat penetration curve for 10,000-foot altitude.

only very slightly the boiling point of water at that altitude. Now the rate and degree of coagulation of the egg and other proteins of the batter and rate and degree of gelatinization of the starch are related to the cooking temperature. Other factors which influence these rates include the water activity, the rate of water evaporation,

the pH of the batter, and specific catalytic influences of certain ingredients. Moreover, to produce the most desirable cake under a given set of external conditions, it is essential that these coagulation and gelatinization reactions shall have proceeded to a sufficient extent at the time the optimum cake volume is produced to furnish the proper amount of structural strength to the cake. Otherwise undue shrinkage or complete collapse will follow. That all these factors must be given consideration in adapting for use at various altitudes a whole-egg cake containing shortening becomes evident.

A baking study of the basic formula established the requirements of this batter for water for different pressure conditions. Less than 180 c.c. of water became impractical at any altitude because the resulting product was too dry. The variation of the water content with altitude is expressed by the following equation:

$$dW = 12\,dA - 0.3\,\overline{dA}^{2}$$

where dW = the change in water requirement in cubic centimeters from the established amount at any base altitude, and dA = the corresponding change in altitude in thousands of feet; dW $\times$ 1.37 = grams weight of whole egg to supply this equivalent amount of water. For sea level the water *requirement* is but 130 c.c., however, 180 c.c. are advisable. This equation applies to altitudes up to and including 10,000 feet.

In order to establish economical and practical limits for the application of this equation, the chart shown (Fig. 10) was built up from the experience gained in an extensive series of baking studies. It was found that this equation was applicable to sugar-rich formulas as well as to the basic recipe. When the requirements for maintenance of a desirable specific volume of the batter were applied, highly desirable and economical cakes resulted; these requirements were discussed in Part I. These cakes are confined to the blocked-in portion of the chart. Outside of this blocked-in portion, cakes may be baked which will not fail, but they will have undesirable features resulting from the limitations in properties of the ingredients used. To the left of the blocked-in portion, the resulting cakes are drier than they should be. To the right of this portion the shrinkage resulting from high liquid content becomes high. Within this blocked-in section the quality of the products is high and the cost per unit volume of cake produced is low.

To apply this chart at any altitude, one selects on the base line the sugar content desired, say 380 grams of sugar per 300 grams of flour. Suppose the altitude in question is 7,500 feet, follow the 380-

gram sugar content vertically until it intersects with a diagonal line which intersects the 7,500-foot line within the blocked-in portion. Three diagonals are found to meet these requirements. The lowest of these three diagonals calls for the use of 240 grams of egg, or five eggs, and 150 grams of shortening. The diagonal intersection on the 7,500-foot line requires 232 c.c. of water. The next higher or middle diagonal of this group would be satisfactory to apply only if the eggs were weighed, since 268 grams of egg are indicated. By this middle diagonal, 155 grams of shortening are required, and the diagonal intersects with the 7,500-foot line at the point calling for 215 c.c. of water. The highest diagonal of the three suggests similarly 288 grams, or six eggs, 160 grams of shortening, and 197 c.c. of water. In other words the sugar, egg, and short-

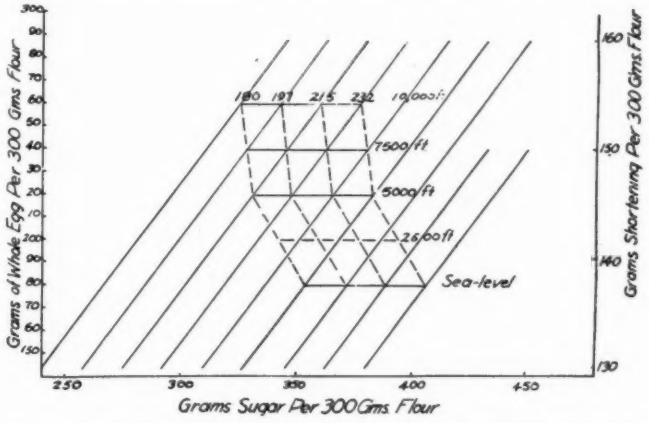


FIG. 10. Nomograph indicating balance of ingredients for whole-egg, butter-type cake for various altitudes.

Grams (c.c.) of water are indicated along the line labeled 10,000 feet and are read downward following the broken lines. All ingredients not indicated on the nomograph are held constant (see text) except the baking powder. The baking powder required may be calculated from the equation $x = 0.5\ A^2 + 4.16A$, where x = per cent decrease in baking powder from the sea-level requirement. (The sea-level requirement for 300 grams of flour in this formula is 12.9 grams.) A = the altitude in thousands of feet. This nomograph has been prepared for use with cake flour and commercial hydrogenated shortening.

ening contents are read off the scales to the bottom, left, and right of the chart, while the water requirement is obtained by following the diagonal corresponding to this "triple point" to where it intersects on the desired altitude line. The cubic centimeters of water are read from the numbers on the corresponding broken line at the top. For convenience these lines represent an increment of water corre-

sponding approximately to half the water content of an egg. The other ingredients are the same as given in the basic formula. The egg substance used in this investigation was always weighed. Forty-eight grams were taken as the weight of the contents of the average egg of which 30 grams were white and 18 grams were yolk.

The advantage of Fig. 10 lies in the fact that it readily permits interpellation to any weight of the corresponding ingredient desired. For the ready reference of those who wish the information in tabular form, Table 7 has been prepared. This gives formulas based on 300 grams of cake flour. For each formula containing over 100 per cent sugar on the flour basis, 105 c.c. of water should be withheld until after eight minutes of mixing. We believe the wire whip to be preferable to any other accepted type of beater.

Reference to Fig. 10 and Table 7 and a consideration of the principles presented in this article should enable any interested person to prepare an indefinite number of whole-egg, butter-type cake formulas for any altitude up to 10,000 feet.

Since egg coagulation at 100°C.(212°F.) is rapid and the product quickly becomes tough and elastic, the egg content of cakes baked at or near sea level must be kept relatively low if desirable products are to be obtained. Sugar has a tenderizing effect upon both the egg coagulum and the starch gel. A cake high in sugar must be kept relatively high in liquid; otherwise neither egg nor starch will set during the baking process. Batters high in liquid and low in egg content show high shrinkage during baking and after removal from the oven. High-quality cakes baked from batters high in sugar and liquid have excellent keeping qualities. This series of facts confronts the baker at low altitudes. As a result the recipes generally adopted for these localities are relatively low in egg content and high in sugar and liquid. The baker dislikes and often complains regarding the high shrinkage he gets under such conditions, but this is the natural price he must pay to obtain the characteristics he desires in his product when the usual ingredients are used.

At altitudes of 5,000 feet and above, the internal temperature obtained when a cake is baked develops a much weaker structure from both the egg and starch ingredients. A batter of much higher egg content than at low altitudes may be baked with advantage if a high sugar ratio is maintained. Naturally the liquid content of such a batter must be suitable for the particular altitude at which it is baked. When such a properly balanced formula is baked at these higher altitudes, a cake of very high quality results. A similar high-egg-ratio batter, properly balanced for egg content, does not produce a desirable product at low altitudes because the higher internal tem-

TABLE 7

Balanced Whole-Egg Cakes With Sugar Content Optional for Altitudes From 0 to 10,000 Feet

(Modified single-stage method of mixing recommended for all but the 92-per cent sugar formula)

Per cent sugar on flour basis.....		92	103	115	127	138	144
Ingredient	Altitude	Gm.	Gm.	Gm.	Gm.	Gm.	Gm.
Cake flour.....	Any	300	300	300	300	300	300
Sugar.....	Any	275	310	345	380	415	432
Dry milk (skim).....	Any	27	27	27	27	27	27
Salt.....	Any	4.5	4.5	4.5	5	5	5
Vanilla.....	Any	5	5	5	5	5	5
Baking powder.....	0	12.9	12.9	12.9	12.9	12.9	12.9
	2,500	11.25	11.25	11.25	11.25	11.25	11.25
	5,000	8.6	8.6	8.6	8.6	8.6	8.6
	7,500	5.2	5.2	5.2	5.2	5.2	5.2
	10,000	1.08	1.08	1.08	1.08	1.08	1.08
Egg white.....	0	90	90	90	120	150	150
	2,500	90	90	120	150	150	180
	5,000 to 10,000	90	120	150	180	180	180
Egg yolk.....	0	54	54	54	72	90	90
	2,500	54	54	72	90	90	108
	5,000 to 10,000	54	72	90	108	108	108
Water.....	0	180	180	197	197	197	215
	2,500	180	190	190	190	225	205
	5,000	180	180	180	180	215	232
	7,500	197	197	197	197	232	250 [1]
	10,000	215	215	215	215	250 [1]	267 [1]
Shortening (hydrogenated).....	0	130	130	130	140	150	150
	2,500	130	130	140	150	150	160
	5,000 to 10,000	130	140	150	160	160	160

[1] Note: Increase of milk solids to double or treble that called for may overcome lack of water tolerance of certain hydrogenated shortenings of low water tolerance.

perature obtained rapidly converts the egg substance into a structure that is too strong and elastic. As a result such cakes show high shrinkage in spite of high egg content. In addition the texture is harsh and undesirable. In contrast, at altitudes of 5,000 and 7,500 feet high-sugar-ratio cakes will tolerate high egg content and at the same time yield highly desirable products with comparatively little shrinkage.

These more highly nutritious cakes are produced just as cheaply, volume for volume, as the somewhat heavier and tougher products now obtained at lower altitudes. They have excellent keeping qualities and are well-balanced food products. As the altitude increases above 7,500 feet, the water requirements to produce the proper set of the baking ingredients continue to increase. At the same time the strength contributed by the coagulated egg and gelatinized starch falls off rapidly. Shrinkage increases again and, volume for volume, costs become greater. However, these high-altitude products are tender, very desirable, and have excellent keeping qualities.

Above 10,000 feet the water requirement of cakes increases more rapidly than indicated by the equation given. At such high altitudes the internal baking temperature of the cake is below 90°C. (194°F.). Both egg and starch, in the presence of sugar, produce very tender gel structures when cooked at such low temperatures. The strength of these structures falls off rapidly as the temperature decreases unless the water ratio is more rapidly increased. Other methods than the increase of the water ratio may be used to increase the strength of the resulting gel structure, but their discussion would be out of place in the present article.

SUMMARY

The principles involved in the maintenance of constant specific volume (within practical limits) for cake batters as the ingredients of the batter are varied, which were presented and discussed in Part I, have been applied in the problem of rebalancing high-sugar-ratio cake formulas for use at various altitudes.

An equation expressing the variation in water of a cake batter as the altitude varies, providing the sugar content remains unchanged, has been presented. The limitations of this formula have been discussed in connection with a whole series of cake formulas designed for use at altitudes varying from sea level to 10,000 feet.

Photographs have been presented showing the results obtained when a high-sugar-ratio cake formula balanced for use at sea level is used at 5,000 and 10,000 feet altitude. The slight improvement in results obtained by applying the baking powder correction is also presented. In comparison, photographs of cakes prepared by following formulas balanced for use at each of these altitudes are shown.

The modified single-stage method of mixing described in Part I was found entirely satisfactory for mixing high-sugar-ratio cakes at high altitudes, where the water requirement is extremely high.

Ordinary hydrogenated commercial cake shortening served satisfactorily in the preparation of balanced cake batters high in liquid when the modified single-stage method of mixing was used.

A nomograph has been presented which permits the formulation of an indefinite number of whole-egg, butter-type cake formulas for any selected altitude up to 10,000 feet. Optional sugar content is permitted.

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ASCORBIC ACID CONTENT OF PARSNIPS[1]

HELEN L. MAYFIELD AND JESSIE E. RICHARDSON
Montana Agricultural Experiment Station, Bozeman, Montana

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Parsnips should be given a more prominent place among the root vegetables on account of certain qualities which they possess. They may either be used in the late fall or left in the ground until spring, when the supply of fresh vegetables is more limited. Exposure to freezing and thawing does not harm the parsnips but improves their flavor and sweetness.

When compared with other vegetables, parsnips possess good nutritive properties. Sherman (1933) showed that base-forming elements predominate to a marked degree in parsnips, which makes them especially valuable for spring use. Very little information about the vitamin values of parsnips has been reported. Sherman (1933) has indicated that they contain a rather small amount of vitamin A but a moderate amount of vitamin B_1. Considerable variation appears in the reports on the vitamin C content of parsnips. McHenry and Graham (1935) have listed them as containing about 3.7 milligrams of reduced ascorbic acid per 100 grams of the raw vegetable and 6.1 milligrams per 100 grams of parsnips boiled 30 minutes. Tauber and Kleiner (1935) show a much higher value, from 36 to 40 milligrams per 100 grams of raw material.

The latter report indicates that parsnips might be a good source of vitamin C. For that reason, this study was carried on to obtain more data concerning the ascorbic acid content of parsnips when raw and when cooked by various standard methods. Tests were made both in the fall and in the spring, after the parsnips had remained in the ground during the winter.

EXPERIMENTAL PROCEDURE

Several lots of parsnips, of the Hollow Crown variety, were purchased at the local market in the fall and in the spring, soon after being dug. They were brought to the laboratory, stored in an electric refrigerator, and tested as rapidly as possible for their ascorbic acid content when raw and when cooked in various ways.

The method of ascorbic acid determination was in general that described by Bessey and King (1933) with a modification in the extracting solution as suggested by Bessey (1938). The method in

[1]Approved by the director of the Montana Agricultural Experiment Station for publication as Journal Paper No. 130, October 26, 1939.

TABLE 1

Ascorbic Acid (A-A) Content of Parsnips Before and After Cooking as Tested in the Fall

Parsnip No.	Weight of parsnip A.P.[1]	A-A content raw parsnip			A-A content cooked parsnip					
					Fresh-boiled parsnip				Boiled parsnip 24 hrs. in refrigerator	
		Top end	Tip end	Mean	Whole, skin on	Peeled, sliced before boiling	Fried at once	Mashed at once	Cold	Fried
Lot I	*gm.*	*mg./gm.*	*mg./gm.*	*mg./gm.*	*mg./gm.*	*mg./gm.*	*mg./gm.*	*mg./gm.*	*mg./gm.*	*mg./gm.*
1	251	.326	.402	.364	.314	.....	.....	.....	.246	.295
2	241	.293	.355	.324	.269	.....	.312	.....	.....	.....
3	303	.205	.293	.249	.223	.....	.....	.....	.203	.225
4	256	.366	.405	.385	.367	.....	.361	.....	.334	.362
5	236	.308	.308	.308	.256	.....	.320	.....	.166	.165
6	284	.201	.382	.292	.243	.....	.277	.....	.151	.164
Mean values	262	.283 ±.071[2]	.357 ±.048	.320 ±.049	.279 ±.053	.....	.318 ±.038	.....	.220 ±.073	.242 ±.086
Lot II										
7	215	Sampled lengthwise		.321	.....	.171	.274	.....	.188	.235
8	163	Sampled lengthwise		.202	.....	.123	.194	.....	.103	.114
9	181	Sampled lengthwise		.187	.....	.114	.158	.....	.124	.157
10	375	Sampled lengthwise		.224	.....	.150	.201	.162	.142	.155
11	410	Sampled lengthwise		.222	.....	.134	.217	.180	.143	.156
12	393	Sampled lengthwise		.211	.....	.154	.....	.167	.....	.....
13	323	Sampled lengthwise		.198	.....	.129	.....	.163	.....	.....
14	404	Sampled lengthwise		.211	.....	.135	.....	.116	.....	.....
Mean values	308	Sampled lengthwise		.222 ±.042	.....	.139 ±.018	.209 ±.042	.158 ±.024	.140 ±.031	.163 ±.044
Lot III										
15	70	Sampled lengthwise		.128	.....	.079				
16	72	Sampled lengthwise		.273	.....	.182				
17	96	Sampled lengthwise		.149	.....	.108				
18	111	Sampled lengthwise		.243	.....	.185				
19	124	Sampled lengthwise		.167	.....	.099				
20	96	Sampled lengthwise		.199	.....	.127				
21	158	Sampled lengthwise		.399	.....	.263				
22	66	Sampled lengthwise		.266	.....	.134				
Mean values	99	Sampled lengthwise		.228 ±.087	.....	.147 ±.060				

[1] A.P.= as purchased. [2] S.D.—calculated according to Snedecor (1937).

brief was as follows: Ten grams of parsnips were triturated twice with acid-washed sand and three per cent metaphosphoric acid. A total of 25 ml. of the acid was used for the two extractions and rinsing, and each time the mixture was filtered through a double layer of cheesecloth. The combined filtrates were made up to a volume of 50 ml. with glass-distilled water. Ten-ml. aliquots of this extract were then titrated with a standardized dye solution (2,6-dichloro-

TABLE 2

Ascorbic Acid (A-A) Content of Parsnips, Kept in the Ground Over Winter, Tested Before and After Cooking

Parsnip No.	Wt. of parsnip A.P.[1]	A-A content raw parsnip			A-A content fresh-boiled parsnip		
		Top end	Tip end	Mean	Whole, skin on	Peeled, sliced before boiling	Fried at once
Lot IV	*gm.*	*mg./gm.*	*mg./gm.*	*mg./gm.*	*mg./gm.*	*mg./gm.*	*mg./gm.*
23	156	.062	.048	.055	.046	.....	.037
24	267	.109	.118	.114	.127	.....	.159
25	137	.096	.158	.127	.124	.....	.144
26	151	.090	.075	.082	.059	.....	.087
27	132	.058	.084	.071	.070	.....	.082
Mean values	169	.083	.097	.090	.085	.....	.102
		±.022	±.042	±.030	±.038	.....	±.050
Lot V							
28	154	Sampled lengthwise		.113	.....	.081	.118
29	146	Sampled lengthwise		.112	.....	.099	.159
30	109	Sampled lengthwise		.083	.....	.059	.071
31	116	Sampled lengthwise		.059	.....	.045	.057
32	110	Sampled lengthwise		.098	.....	.071	.105
33	95	Sampled lengthwise		.077	.....	.044	.078
Mean values	122	Sampled lengthwise		.090	.....	.067	.098
				±.021	.....	±.021	±.037

[1] A.P.= as purchased.

phenolindophenol) from a micro-burette. Determinations were also made on blanks containing only the acid in a similar volume.

Ascorbic Acid Content of Raw Parsnips: Two methods were used for obtaining the raw samples needed for ascorbic acid determinations. Some of the parsnips were to be boiled whole with skins on, so the raw samples were cut from the top and tip ends, peeled, and tested separately. The rest of the parsnips were to be peeled and sliced lengthwise for boiling, so the raw samples were cut as lengthwise, wedge-shaped sections.

The ascorbic acid values for the respective samples of individual parsnips from five different lots purchased in fall and spring are presented (Tables 1 and 2). In Lot I, tested in the fall, higher val-

ues were found in the tip end than in the upper portion of the root. This may have been due to a region of greater growth activity near the tip end. In Lot IV, the same condition did not always exist, probably for the reason that the roots had been in the frozen ground during the winter. In both lots, the ascorbic acid values for the two ends of each root were averaged to obtain an estimated value for the entire parsnip.

The parsnips in Lots II and III were sampled lengthwise and it will be observed that they show ascorbic acid values somewhat lower than those estimated from top and tip end tests, in Lot I. Apparently this difference should not be attributed to the method of sampling, but rather to the fact that parsnips purchased in different lots show some variation in ascorbic acid content. This explanation is supported by data on cooked parsnips (Table 1).

There seems to be considerable variation in the ascorbic acid values per unit weight among the individual parsnips. There also is great variation in the weights of the parsnips (Tables 1 and 2), but when these two factors are compared, no relationships are evident. Parsnips tested in the spring, after being in the frozen ground during the winter, have much lower ascorbic acid values than in the fall (Table 2). They appear to have lost about 60 to 70 per cent of their vitamin C content.

Ascorbic Acid Content of Boiled Parsnips: Each parsnip was prepared for cooking, weighed, and boiled by itself in salted tap water in a covered aluminum saucepan. Some of the parsnips were boiled whole with skins left on and were tender in about 30 minutes. Some were peeled and sliced lengthwise for boiling and were cooked from 20 to 25 minutes. When the parsnips were done, they were drained and weighed. A representative lengthwise sample was then cut from each parsnip, peeled if necessary, and tested for its vitamin C content.

The parsnips boiled whole with skins on lost only a small portion of their original ascorbic acid content, about 13 per cent (Table 1, Lot I). Those that were peeled, sliced, and then boiled lost about 36 per cent of their original content (Table 1, Lots II and III). It is believed that this difference in losses of ascorbic acid is due only to the different conditions of the parsnips during boiling, for the slight weight changes that occurred were rather similar for both groups.

In the spring, when the ascorbic acid content of the raw parsnips was much lower, there was very little loss when the parsnips were boiled whole, but about 26 per cent loss when they were peeled and sliced before boiling (Table 2, Lots IV and V). However, the rela-

tive difference between the ascorbic acid values of the parsnips boiled whole and those boiled after peeling and slicing was about the same in both fall and spring.

Ascorbic Acid Content of Mashed Parsnips: Since boiled parsnips are sometimes mashed before serving, a few tests were made to determine the effect of mashing on their ascorbic acid content.

Portions of hot, fresh-boiled parsnips were thoroughly mashed and whipped for one minute. The sample was weighed out and tested at once.

The individual records for five parsnips thus treated (Table 1, Lot II) show that there is a tendency for the mashed parsnips to show a slightly higher ascorbic acid value than when they were tested in the boiled form.

Ascorbic Acid Content of Fried Parsnips: Another common method of preparing parsnips for table use is to place slices of boiled parsnips in a small amount of hot butter and fry them until nicely browned. For this reason some additional tests were made to determine the ascorbic acid value of fried parsnips.

A weighed portion of sliced, fresh-boiled parsnip was placed in an aluminum frying pan with a weighed amount of hot butter. It was allowed to fry without scorching for about six minutes, at which time all the butter was absorbed. The fried parsnip was then weighed and a 10-gram sample was carefully selected from the various pieces. This was tested for its vitamin C content.

In some cases the boiled parsnips were wrapped in waxed paper, placed in an electric refrigerator for 24 hours, and fried the following day in a similar manner. Tests were made for ascorbic acid content both before and after frying. Data on the ascorbic acid content of fried parsnips taken from fall and spring lots are presented (Tables 1 and 2).

These data indicate that when the parsnips had been boiled whole with skins on and fried soon after (Lots I and IV), the ascorbic acid value of the fried material was, in general, slightly higher than that of the boiled. When the parsnips had been peeled and sliced before boiling and then fried (Lots II and V), there was evidence of still higher ascorbic acid values for the fried material as compared with the boiled.

It was also observed that when boiled parsnips were kept in the refrigerator 24 hours and then fried, the ascorbic acid content of the fried material was also slightly higher than that of the cold, stored parsnips tested just before frying. Incidentally, the ascorbic acid value of these fried parsnips was not as high as for parsnips fried soon after boiling.

TABLE 3

Successive Weight Changes of Individual Parsnips, Boiled Then Fried, and Their Total Ascorbic Acid (A-A) Content

Parsnip No.	Raw		Boiled whole		Fried at once		Parsnip No.	Raw		Boiled, peeled, and sliced		Fried at once	
	Weight	A-A content	Weight	A-A content	Weight	A-A content		Weight	A-A content	Weight	A-A content	Weight	A-A content
Tested in fall	*gm.*	*mg.*	*gm.*	*mg.*	*gm.*	*mg.*	Tested in fall	*gm.*	*mg.*	*gm.*	*mg.*	*gm.*	*mg.*
2	100 [1]	32.4	108	29.0	91	28.3	7	100 [1]	32.1	115	19.7	99	27.1
4	100	38.5	108	39.6	95	34.3	8	100	20.2	119	14.6	103	20.0
5	100	30.8	105	26.9	86	27.5	9	100	18.7	105	12.0	88	13.9
6	100	29.2	109	26.5	95	26.3	10	100	22.4	111	16.6	95	19.1
							11	100	22.2	104	13.9	96	20.8
Mean	100	32.7	107.5	30.5	91.7	29.1	Mean	100	23.1	110.8	15.3	96.2	20.2
Tested in spring							Tested in spring						
23	100 [1]	5.5	95	4.4	81	3.0	28	100 [1]	11.3	108	8.7	92	10.8
24	100	11.4	101	12.8	86	13.7	29	100	11.2	100	9.9	73	11.6
25	100	12.7	102	12.6	83	11.9	30	100	8.3	112	6.6	83	5.9
26	100	8.2	88	5.2	75	6.5	31	100	5.9	109	4.9	79	4.5
27	100	7.1	90	6.3	74	6.1	32	100	9.8	100	7.1	72	7.6
							33	100	7.7	105	4.6	83	6.5
Mean	100	9.0	95.2	8.3	79.8	8.2	Mean	100	9.0	105.7	7.0	80.3	7.8

[1] Recalculated on a 100-gram basis.

DISCUSSION

The ascorbic acid content of the raw parsnips varied considerably. In the fall these values ranged from 12.8 to 40 milligrams of ascorbic acid per 100 grams of the edible portion of the raw material. In the spring, similar tests gave results ranging from 5.5 to 12.7 milligrams. When compared with the vitamin C values of parsnips previously reported, it is of interest to note that the fall values agree fairly well with the data shown by Tauber and Kleiner (1935), while the spring values more closely resemble those of McHenry and Graham (1935).

When the parsnips were cooked, the changes in their ascorbic acid content showed some consistent trends. Upon boiling, the parsnips

TABLE 4

Ascorbic Acid Content of One Serving of Cooked Parsnips

Condition of cooked parsnips	Ascorbic acid content of a 70-gram serving	
	Fall	Spring
	mg.	*mg.*
A. Boiled whole—skins left on	15	6
Fried at once	18	7
In refrig. 24 hours	12	....
Fried after stored in refrig. 24 hours	14	....
B. Boiled after peeling and slicing	11	5
Fried at once	16	7
Mashed	13	....
In refrig. 24 hours	11	....
Fried after stored in refrig. 24 hours	12	....

sustained certain losses in ascorbic acid content; but when subjected to a second period of cooking by frying, there were evidences of increased ascorbic acid values.

As this increase might be attributed to loss of moisture during frying and hence a greater concentration of vitamin C in the fried material, some information on this point has been assembled (Table 3) showing the weight changes that occurred during the successive boiling and frying of individual parsnips. Using 100 grams of raw parsnips as a basis, the corresponding weights when boiled and fried were calculated. Then by referring to the ascorbic acid values for each parsnip at each stage of cooking (Tables 1 and 2), it was possible also to calculate the total ascorbic acid content of the respective portions. When the changes in moisture content are considered, there appear to be only small losses of ascorbic acid

when parsnips are boiled whole and fried, both in the fall and spring. However, when parsnips are peeled, sliced, then boiled and fried, there seems to be a marked loss of ascorbic acid on boiling but a regain upon frying.

Other nutrition workers have reported many cases in which higher ascorbic acid values were found for the food in its cooked condition. These results and several explanatory theories have been well summarized by Fixsen (1938). None of these theories seems to apply to this case, however, where the increase occurs with the second cooking. There is a possibility that during this cooking certain substances are formed that have dye-reducing properties, Borsook, Davenport, Jeffries, and Warner (1937) and Mack and Tressler (1937).

In order that these varied data on the ascorbic acid content of cooked parsnips may be more readily used for dietary calculations, they have been summarized (Table 4) to show the approximate number of milligrams of ascorbic acid in *one serving* (70 grams).

CONCLUSIONS

1. In the fall, parsnips cooked by various methods are a good source of vitamin C.

2. Parsnips which have remained in the ground over winter are still a fair source of vitamin C, when cooked.

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A STUDY OF CONCENTRATION AND FREEZING AS A MEANS OF PRESERVING FLUID WHOLE MILK[1]

R. T. CORLEY AND F. J. DOAN
Pennsylvania State College, State College, Pennsylvania

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The concentration and freezing of whole milk has been advocated as a treatment having possibilities in the preservation of milk for future use after a period of holding, storage, or transportation. Webb and Hall (1935) reported on some of the physical effects of freezing milk and cream and concluded that fresh milk could be pasteurized, condensed to one-third its weight, canned, and frozen without any detrimental effects to the body and flavor of the product. When held frozen and then reconstituted within a four-week period, a good-quality fluid milk resulted. McBride (1937) has given a résumé of the frozen milk process and its future possibilities. Doan and Featherman (1937) found that concentrated milk, to be frozen and stored, should be concentrated at a ratio of 3 to 1, and should be homogenized. The thawing temperature was not found to be important, but milk processed in a copper vacuum pan invariably became tallowy during storage although homogenization appeared to partially inhibit the development of the flavor. Bell (1939) stated that when the concentrated milk was heated to 76.5°C. (170°F.) and held eight minutes as pasteurization treatment, homogenized at 2,000 to 3,000 pounds, and stored at —7°C. (19.5°F.) best results in the manufacture of frozen, concentrated whole milk were obtained.

Studies with frozen, concentrated skim milk have been reported by Reichart and Corley (1938), Openlander and Erb (1938), and Reichart (1938).

In the study reported here, attempts were made to ascertain the effect of copper vacuum pans compared with stainless steel pans on the quality of the concentrated, frozen milk after storing for various periods, thawing, and reconstituting. In similar fashion the effects of pasteurizing treatment and the homogenizing process were also noted and the maximum time the product could be held in the frozen condition was determined.

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EXPERIMENTAL PROCEDURE

Fresh, fluid, mixed milk of good quality was taken for the preparation of the concentrated samples. Each lot was divided into three portions. These portions were then treated as follows:

A. The first portion was pasteurized at 62.8°C.(145°F.) for 30 minutes, condensed 3 to 1, frozen, and held at —17.8°C.(0°F.).

B. The second portion was pasteurized at 145°F. for 30 minutes, condensed 3 to 1, homogenized at 2,500 pounds, frozen, and held at 0°F.

C. The third portion was pasteurized at 145°F. for 30 minutes, homogenized at 2,500 pounds pressure, condensed 3 to 1, frozen, and held at 0°F.

The three lots (A, B, and C), after condensing and cooling and before freezing, were standardized to exactly the same total solids content so that they were identical in composition. A sufficient number of two-quart tins from each lot, to allow for a 5-week, a 10-week, and a 15-week examination, were frozen and held.

Processing was carried out in both copper and stainless steel pans.

The procedure described above was repeated with other lots of milk, using a pasteurization treatment of 82.2°C.(180°F.) for 15 minutes. In this group, each lot of milk was divided into the three portions (A, B, and C) and processed as outlined previously, again using the copper and stainless steel pans.

As a result of previous experimental work on the subject of frozen, concentrated milk, it was decided to study the effect of freezing and holding of concentrated milk on the following characteristics of the reconstituted milk: flavor, de-emulsification of fat, alcohol stability, and curd tension. The samples of condensed milk were also examined after thawing and before reconstitution for flavor and the presence of flaking, coagulation, or gelation.

De-emulsification of fat occurring as a result of freezing and holding of concentrated whole milk was determined by a method similar to that used by Webb and Hall except that Babcock milk test bottles were used and the samples were examined after reconstitution.

EFFECT OF PASTEURIZATION TREATMENT ON SOME PROPERTIES OF STORED, FROZEN, CONCENTRATED WHOLE MILK

The data reveal that differences in pasteurization treatment affect, to some extent, the flavor of the concentrated whole milk after freezing and storing for periods of 5, 10, and 15 weeks (Tables 1, 2, and 3). It was observed that the higher temperature treatment caused a cooked flavor to persist in a number of the samples for 5 and 10 weeks of storage, but only in cases where the product was condensed

in a stainless steel pan. At the end of 15 weeks of storage no cooked flavors were observable. Tallowy flavor appeared in all the samples made in the copper pan. It was evident sooner or to a greater degree in the unhomogenized samples and in the samples pasteurized at the lower temperatures. No diminution of the tallowy flavor during storage was noted in any of the samples as was evident in Bell's (1939) data. In fact tallowy flavor appeared to increase in degree during storage.

Differences in pasteurization treatment of the milk prior to condensing apparently has some influence on the body of the product after storage. At the end of five weeks of storage at 0°F., the samples that had been given the low-temperature pasteurization treatment were all in a liquid condition after thawing, although two of the 12 examined showed the presence of a slight gelation. On the other hand, the samples given the high-temperature pasteurization treatment varied in condition from a smooth, viscous appearance to a heavy gel. Of the 12 samples in this group more than half displayed a moderate to heavy gelation. Apparently, high preheating temperatures favor gelation on storage as is true in the case of sweetened condensed milk.

At the end of 10 weeks storage nearly all of the samples showed definite gelation, while at 15 weeks some displayed a syneresis or wheying-off effect. This particular effect was not confined to samples of milk pasteurized by either one of the two treatments used but appeared to be more pronounced in the samples which were not homogenized.

Doan and Fetherman (1937) previously indicated that the gelation and flocculation caused by holding concentrated milk in the frozen condition for periods over four weeks was capable of being reversed by heating momentarily to temperatures of 65.6 to 71.1°C. (150 to 160°F.).

The data (Tables 1, 2, and 3) reveal that where flocculation or gelation occurred upon thawing of the frozen, concentrated milk, the coagulated protein was most readily redispersed by heat in those samples subjected to the high temperature of pasteurization. Evidence is presented (Table 2) that homogenization after condensation was better practice than homogenization before condensation in that the period of storage possible before the appearance of a non-redispersible coagulation was increased.

It thus appears that a pasteurization treatment using high temperature and homogenization after condensation, makes possible a longer storage period for concentrated, frozen milk.

The amount of fat thrown out of emulsion as indicated (Tables 1, 2, and 3) increased as the storage period lengthened, was slightly

TABLE 1

Effect of Pasteurization Treatment on Some Properties of Frozen, Concentrated Whole Milk After a Storage Period of Five Weeks at —17.8°C.(0°F.) and Reconstitution

Lot No.		Flavor	Appearance when thawed	Treatment of coagulation	Fat de-emulsified (pct.)
Pasteurized at 62.8°C.(145°F.) for 30 Minutes					
1	A[1]	Tallowy^{+++}	Sl. gelation	Not redispersible	.10
	B	Tallowy^{++}	Sl. gelation	Redispersed by heat	None
	C	Tallowy^{+}	Fluid; very viscous	None required	None
2	A	Tallowy^{++}	Fluid; smooth	None required	.50
	B	Tallowy^{+}	Fluid; smooth	None required	None
	C	Tallowy^{+}	Fluid; smooth	None required	None
3	A	Sl. tallowy	Smooth; viscous	None required	.45
	B	Good	Smooth; viscous	None required	None
	C	Good	Smooth; viscous	None required	None
4	A	Good	Smooth; viscous	None required	.15
	B	Good	Smooth; viscous	None required	None
	C	Good	Smooth; viscous	None required	.15
Pasteurized at 82.2°C.(180°F.) for 15 Minutes					
5	A	Sl. tallowy	Sl. flocculation	Redispersed by heat	.50
	B	Good	Smooth; viscous	None required	None
	C	Good	Smooth; viscous	None required	None
6	A	Tallowy^{+}	Viscous	None required	.35
	B	Sl. tallowy	Viscous	None required	None
	C	Sl. tallowy	Viscous	None required	None
7	A	Tallowy^{+}	Heavy gel	Redispersed by heat	.65
	B	Sl. tallowy	Heavy gel	Redispersed by heat	None
	C	Sl. tallowy	Heavy gel	Redispersed by heat	None
8	A	Cooked; flat	Partial gel	Redispersible by heat	.60
	B	Cooked; flat	Very viscous	Redispersed by heat	None
	C	Cooked; flat	Partial gel	Redispersed by heat	None

[1] 1—Concentrated in copper vacuum pan; 2—Concentrated in copper vacuum pan; 3—Concentrated in stainless steel vacuum pan; 4—Concentrated in stainless steel vacuum pan; 5—Concentrated in copper vacuum pan; 6—Concentrated in copper vacuum pan; 7—Concentrated in copper vacuum pan; 8—Concentrated in stainless steel vacuum pan.

A—Pasteurized, condensed 3 to 1, frozen, and stored; B—Pasteurized, condensed 3 to 1, homogenized, frozen, and stored; C—Pasteurized, homogenized, condensed 3 to 1, frozen, and stored.

TABLE 2

Effect of Pasteurization Treatment on Some Properties of Frozen, Concentrated Whole Milk After a Storage Period of Ten Weeks at —17.8°C.(0°F.) and Reconstitution

Lot No.		Flavor	Appearance when thawed	Treatment of coagulation	Fat de-emulsified (pct.)
Pasteurized at 62.8°C.(145°F.) for 30 Minutes					
1	A[1]	Tallowy^{+++}	Gelation	Not redispersible	.15
	B	Tallowy^{++}	Gelation	Redispersible by heat	.05
	C	Tallowy^{++}	Flocculation	Not redispersible	.05
2	A	Tallowy^{++}	Gelation	Not redispersible	.60
	B	Tallowy^{++}	Flocculation	Redispersible by heat	.05
	C	Tallowy^{+}	Gelation	Not redispersible	.05
3	A	Sl. tallowy	Gelation	Not redispersible	.50
	B	Good	Gelation	Redispersible by heat	None
	C	Good	Gelation	Not redispersible	.05
4	A	Sl. off	Gelation	Not redispersible	.35
	B	Good	Smooth; viscous	None required	None
	C	Good	Gelation	Redispersible with difficulty	.05
Pasteurized at 82.2°C.(180°F.) for 15 Minutes					
5	A	Tallowy^{+++}	Very viscous; partial gelation	Not redispersible	.75
	B	Tallowy^{+}	Very viscous; partial gelation	Redispersed by heat	.05
	C	Tallowy^{+}	Very viscous; partial gelation	Redispersed by heat	.05
6	A	Tallowy^{+++}	Very viscous	None required	.50
	B	Tallowy^{++}	Very viscous	None required	None
	C	Tallowy^{+++}	Very viscous	None required	.05
7	A	Tallowy^{+++}	Heavy gelation	Redispersed by heat	.70
	B	Tallowy^{++}	Heavy gelation	Redispersed by heat	.10
	C	Tallowy^{++}	Heavy gelation	Redispersed by heat	.15
8	A	Cooked; flat	Heavy gelation	Redispersed by heat	.65
	B	Cooked; flat	Heavy viscous	None required	None
	C	Cooked; flat	Heavy gelation	Redispersed by heat	.05

[1] 1—Concentrated in copper vacuum pan; 2—Concentrated in copper vacuum pan; 3—Concentrated in stainless steel vacuum pan; 4—Concentrated in stainless steel vacuum pan; 5—Concentrated in copper vacuum pan; 6—Concentrated in copper vacuum pan; 7—Concentrated in copper vacuum pan; 8—Concentrated in stainless steel vacuum pan.

A—Pasteurized, condensed 3 to 1, frozen, and stored. B—Pasteurized, condensed 3 to 1, homogenized at 2,500 pounds pressure, frozen, and stored; C—Pasteurized, homogenized at 2,500 pounds pressure, condensed 3 to 1, frozen, and stored.

TABLE 3

Effect of Pasteurization Treatment on Some Properties of Frozen, Concentrated Whole Milk After a Storage Period of Fifteen Weeks at —17.8°C.(0°F.) and Reconstitution

Lot No.		Flavor	Appearance when thawed	Treatment of coagulation	Fat de-emulsified (pct.)
PASTEURIZED AT 62.8°C.(145°F.) FOR 30 MINUTES					
1	A[1]	Tallowy^{+++}	Gelation; syneresis	Not redispersible	.25
	B	Tallowy^{++}	Gelation	Not redispersible	.10
	C	Tallowy^{++}	Flocculation	Not redispersible	.15
2	A	Tallowy^{+++}	Gelation; syneresis	Not redispersible	.70
	B	Tallowy^{+++}	Gelation	Not redispersible	.05
	C	Tallowy^{+++}	Gelation	Not redispersible	.10
3	A	Tallowy	Gelation	Not redispersible	.75
	B	Flat	Gelation	Not redispersible	.05
	C	Flat	Gelation	Not redispersible	.10
4	A	Flat	Gelation	Not redispersible	.50
	B	Flat	Sl. flocculation	Redispersed by heat	.05
	C	Flat	Gelation	Not redispersible	.15
PASTEURIZED AT 82.2°C.(180°F.) FOR 15 MINUTES					
5	A	Tallowy^{+++}	Gelation; syneresis (pronounced)	Redispersed by heat	.80
	B	Tallowy^{++}	Gelation; syneresis	Redispersed by heat	.10
	C	Tallowy^{++}	Gelation; syneresis	Redispersed by heat	.20
6	A	Tallowy^{+++}	Very viscous	None required	.60
	B	Tallowy^{++}	Very viscous	None required	.05
	C	Tallowy^{++}	Very viscous	None required	.05
7	A	Tallowy^{+++}	Heavy gelation	Redispersed by heat	.80
	B	Tallowy^{+++}	Heavy gelation	Redispersed by heat	.10
	C	Tallowy^{+++}	Heavy gelation	Redispersed by heat	.10
8	A	Flat	Heavy gelation	Redispersed by heat	.80
	B	Flat	Heavy gelation	Redispersed by heat	.10
	C	Flat	Heavy gelation	Redispersed by heat	.10

[1] 1—Concentrated in copper vacuum pan; 2—Concentrated in copper vacuum pan; 3—Concentrated in stainless steel vacuum pan; 4—Concentrated in stainless steel vacuum pan; 5—Concentrated in copper vacuum pan; 6—Concentrated in copper vacuum pan; 7—Concentrated in copper vacuum pan; 8—Concentrated in stainless steel vacuum pan.

A—Pasteurized, condensed 3 to 1, frozen, and stored. B—Pasteurized, condensed 3 to 1, homogenized at 2,500 pounds pressure, frozen, and stored; C—Pasteurized, homogenized at 2,500 pounds pressure, condensed 3 to 1, frozen, and stored.

greater for the samples pasteurized at high temperature, was greatly decreased by homogenization, and was less for the samples homogenized after condensation than for those homogenized prior to condensation. The quantity of fat de-emulsified in the unhomogenized samples was always an objectionable feature.

INFLUENCE OF CONDENSING, FREEZING, AND STORAGE ON STABILITY OF PROTEIN OF WHOLE MILK

The data show that the protein of most of the milk samples was coagulated or gelated after condensing, freezing, holding for periods of 10 weeks and over at —17.8°C.(0°F.), and thawing (Tables 1, 2, and 3). The gelation became more pronounced on longer holding. Up to a certain point in the storage period the coagulation or gelation was reversible by heating the thawed, concentrated milk, either before or after reconstitution, to temperatures in the neighborhood of 65.6°C.(150°F.). Longer holding in the frozen condition, however, rendered the protein coagulation non-reversible. It was considered of interest to study the protein stability of the milk on a reconstituted basis, using the alcohol number, at various stages of the processing and holding (Table 4). The findings show that as long as the protein coagulation or gelation was dispersible on heating, the stability toward alcohol was not significantly affected by holding in the frozen condition. When the coagulation could no longer be reversed, alcohol tests could not be read accurately. It is apparently possible to preserve milk for periods considerably longer than suggested by Webb and Hall (1935) and Bell (1939) since the initial coagulation of the protein encountered upon holding the concentrated product in the frozen condition is reversible and the appearance and properties of the reconstituted milk are apparently normal so long as this is true.

INFLUENCE OF CONDENSING, FREEZING, AND STORAGE ON DIGESTIBILITY OF WHOLE MILK

Data obtained in this study showed that the curd tension of the processed milk before freezing and after various intervals of storage in the frozen condition was always within five grams of zero, and over half the determinations exhibited no tension. Since these results were so similar to those commonly obtained with evaporated milk, it was considered interesting to determine the *in vitro* digestibility of the concentrated, frozen, stored, and reconstituted milk to note whether the digestibility is also comparable. Accordingly, digestion trials were made using the method of Doan and Flora (1939). The results of a typical trial (Fig. 1) show that processed, reconsti-

TABLE 4

Influence of Pasteurization, Concentration, Freezing, and Storage on Alcohol Stability of Whole Milk After Reconstitution

Lot No.		Ml. alcohol to coagulate 5 ml. of milk			
		Raw fluid milk	Pasteurized, concentrated, and frozen		
			After 5 weeks' storage	After 10 weeks' storage	After 15 weeks' storage
Pasteurized at 62.8°C.(145°F.) for 30 Minutes					
1	A[1]	8.5	....[2]	....	....
	B	8.5	9.5	9.5	....
	C	8.5	9.5	....	....
2	A	8.5	9.5	....	....
	B	8.5	9.5	9.5	....
	C	8.5	9.5	....	....
3	A	8.5	9.5	....	....
	B	8.5	9.5	9.5	....
	C	8.5	9.5	....	....
4	A	8.25	9.5	....	....
	B	8.25	9.75	9.75	9.75
	C	8.25	9.5	9.5	....
Pasteurized at 82.2°C.(180°F.) for 15 Minutes					
5	A	8.0	Not made	....	9.0
	B	8.0	Not made	9.0	9.0
	C	8.0	Not made	9.0	9.0
6	A	8.0	Not made	9.0	9.0
	B	8.0	Not made	9.0	9.0
	C	8.0	Not made	9.0	9.0
7	A	8.5	9.5	9.5	9.5
	B	8.5	9.5	9.5	9.5
	C	8.5	9.5	9.5	9.5
8	A	8.5	9.5	9.5	9.5
	B	8.5	9.5	9.5	9.5
	C	8.5	9.5	9.5	9.5

[1]A—Unhomogenized; B—Homogenized after condensing; C—Homogenized before condensing. [2]Where no figure is given, the coagulation owing to storage could not be satisfactorily redispersed by heating to 65.6°C.(150°F.).

tuted milk digested at a more rapid rate than fresh fluid milk but not so rapidly as the milk which had been frozen and stored. The curve obtained with the latter showed the typical feature exhibited by evaporated milk, acidified milk, and some samples of boiled milk in that it started high and dropped in the peptic period. The heated samples exhibited less "soluble" nitrogen at three hours but more "soluble" nitrogen after trypsin action at six hours, in accord with the findings of Doan and Flora (1939) for heated fluid milk.

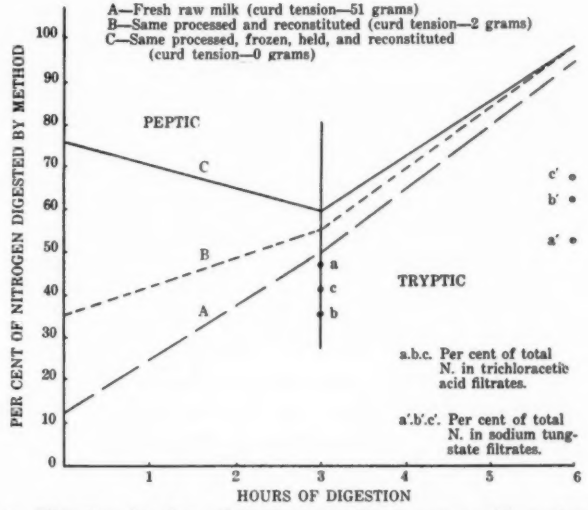
A—Fresh raw milk (curd tension—51 grams)
B—Same processed and reconstituted (curd tension—2 grams)
C—Same processed, frozen, held, and reconstituted (curd tension—0 grams)
PEPTIC
TRYPTIC
a.b.c. Per cent of total N. in trichloracetic acid filtrates.
a'.b'.c'. Per cent of total N. in sodium tungstate filtrates.
PER CENT OF NITROGEN DIGESTED BY METHOD
HOURS OF DIGESTION

FIG. 1. Digestion *in vitro* of fresh raw milk, the same milk pasteurized at 82.2°C.(180°F.) for 30 minutes, concentrated, homogenized and reconstituted and the same milk similarly processed, frozen, and stored five weeks at —17.8°C. (0°F.) and reconstituted.

CONCLUSIONS

While a pasteurizing treatment at 62.8°C.(145°F.) retards the rate of thickening of concentrated, frozen milk during storage compared with a pasteurizing treatment at 82.2°C.(180°F.), the latter temperature is preferable since it retards the development of tallowy flavors and lessens the tendency for the formation of irreversibly coagulated protein. Therefore the higher pasteurization temperature makes possible longer storage periods.

Homogenization retards the development of tallowy flavor during storage of concentrated, frozen milk, lessens the rate of thickening, postpones the appearance of an irreversible coagulation, and aids in maintaining the fat in an emulsified condition. The three

last-mentioned effects are more definite when homogenization follows condensation than when homogenization precedes condensation.

If milk is contaminated with copper it appears impossible to avoid tallowy flavor, even with high pasteurizing temperatures and homogenization, unless the storage period in the frozen condition is less than five weeks.

Even though a coagulation or flocculation of the protein of concentrated milk forms during storage in the frozen condition, if this is capable of being redispersed by heating the thawed, concentrated milk or the thawed, reconstituted milk, the protein stability toward alcohol is not affected and the properties of the milk appear to be normal.

An irreversible coagulation or flocculation of the proteins occurs only after the frozen, concentrated milk has been held in storage for periods of 15 weeks or more, provided the milk has been pasteurized at high temperature (180°F.) and homogenized after condensing.

Concentrated, frozen, held, thawed, and reconstituted milk exhibits *in vitro* digestion characteristics superior to boiled milk and somewhat similar to evaporated and acidified milk.

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SOME MODIFICATIONS OF THE PAIRED-EATING METHOD IN MEAT COOKERY RESEARCH [1]

SYLVIA COVER
Texas Agricultural Experiment Station,
College Station, Texas

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The paired-eating method described previously by Cover (1936) has recently been modified in several important respects so that it is now a more trustworthy tool. Since it has proved valuable in meat cookery research, the suggestion has been made that it also may serve a useful purpose in cookery research with other foods wherever suitable conditions may be maintained.

All cookery research is concerned with the nature of the changes taking place in food as it is being prepared for the table. That phase of cookery research which is concerned especially with palatability seeks to reveal the causes of changes, taking place in food after it is in the kitchen, which affect palatability so that advantage may be taken of them in stating directions for cooking in order that the food, when served, will *always* be as high as possible in *all* the desirable qualities of palatability.

There are many factors of palatability in meat and in other foods. There are also many environmental factors which influence each of these factors of palatability. Such a situation calls for an experiment of complicated design, but lack of satisfactory quantitative measures for the various factors of palatability make a design of this sort impracticable at the outset of research. Also, when many variables are present for which adequate quantitative tests are not available, a study of all of them at once is likely to distract the worker so that only superficial consideration will be given to any one variable. When little information is available on the mode of action of any independent variable on any dependent variable, it would seem wise to establish these relationships first by experiments of simple design. This may be done (as with meat) by limiting each study to only one dependent variable (such as tenderness) and only one independent variable (such as oven temperature). Experiments of such simple design may often be planned to furnish situations in which direct comparisons are possible. In such situations the paired-eating method is applicable.

[1]Approved by the director of the Texas Agricultural Experiment Station for publication as Technical Contribution No. 556, October 26, 1939.

In meat, tenderness is, perhaps, the quality of palatability which consumers consider most important. After many years of research, however, we are not yet able to give satisfactory directions for cooking meat so that it will always be tender. There is some information on the effect of oven temperature and time of cooking on the tenderness of meat, but such other factors as pressure, air velocity, and moisture in the surrounding air during cooking, have received little attention. Perhaps separate controlled studies using each of these physical factors as an independent variable would help to clarify the problem of cooking meat for tenderness. Similar problems would, no doubt, be found for flavor and for each other factor of palatability when it is considered as a single dependent variable. When we can be certain what are the important factors influencing tenderness alone or flavor alone or any other individual factor of palatability, progress will have been made toward controlling the palatability of meat.

Suitable equipment is a puzzling problem. At the present time, it is not only impossible to control all of the physical factors in the surroundings of meat during cooking, but these factors cannot even be measured inside an oven where a roast is cooking. It seems preferable, therefore, to study only one of these independent variables at one time so that equipment may be designed for obtaining suitable conditions for investigation.

It should be remembered that tenderness (or any other factor of palatability) may be influenced by several physical conditions in the cooking process and that the effect of any one of them is likely to be small. It would seem, therefore, only logical to plan an experiment in such a way that the extremes of the variable are tested first. Then, if significant differences are found between the two extremes and if gradations between the extremes are desirable, the gradations may be studied later.

As the differences in tenderness (or any other factor of palatability) are likely to be small with a single variable, the method of testing used should be one which is capable of detecting small differences.

It should, also, provide the conditions necessary for the application of reasonably simple statistical analyses to the data obtained.

The paired-eating method was designed to meet these requirements. The discussion will be limited to the use of the method in meat cookery research, but its application to cookery research with other foods should be apparent. Only slight changes in the forms would be necessary.

DESCRIPTION OF PAIRED-EATING METHOD AS USED IN TESTING TENDERNESS OF MEAT

The essential and distinctive features of the paired-eating method are (1) the providing of paired samples and (2) the judging of the paired samples by comparison with each other. *For the paired samples*, only two roasts are used in each test and should be corresponding cuts from the right and left sides of the same animal. They should differ from each other in only one important variable or treatment. An interior slice from one roast is paired with an interior slice from the same position in the other roast. Paired samples are obtained from each pair of slices by cutting them from corresponding places in the same muscle of each slice. Each sample must be small enough to be eaten in one mouthful. *For judging*, the paired samples from paired slices from paired roasts are given to judges who eat them and rank them for tenderness. The judges check on record sheets their decision as to whether or not there is a difference in tenderness and, if so, which sample is the more tender.

It is, of course, necessary that the judges be free to give an unbiased opinion; to do this, they must be prevented from identifying their samples. Improvements in this respect have been made frequently during the past four years. With the modification in use at present all of the judges may sit around the same table at once and may talk freely about their samples, but no one of them will be able to identify his samples with those of his neighbor or of anyone else at the table. Nor will he be able to identify them with the roasts from which they came. This method of sampling requires a sampling sheet which must be made out before the time for judging. The sampling sheet is really a code which is followed in placing the samples on the plates of the individual judges.

The placing of the samples in their proper positions on the plate is a rather complicated procedure and requires a high degree of concentration. It is suggested that a separate plate and separate sheets be used for successive pairs of roasts and that the judges assemble before the plates are ready. The time elapsing between cutting and testing will then be as short as possible. Numbers are written on each plate with a glass marking pencil to designate the position of each sample. Thus, if four paired slices are used from one pair of roasts, eight samples will be given to each judge. Accordingly, the numbers 1 to 8 are written on the rim of each plate with 1 and 2 close together, 3 and 4 together, 5 and 6 together, and 7 and 8 together. The judges must know that those samples which are placed close together are pairs.

In preparing the sampling sheet (Fig. 1) the "judge" number

SAMPLING SHEET

Roast numbers	667		667					
Letters denoting position of paired slices	A		B		C		D	
Roast identification	*L*	*R*	*L*	*R*				
Basis of pairing	*Method I*	*Method II*	*Method I*	*Method II*				
Judge	Sample number on plate							
1. *A.K.M.*	*1*	*2*	*4*	*3*				
2. *C.E.M.*	*1*	*2*	*3*	*4*				
3. *P.B.P.*	*1*	*2*	*4*	*3*				
4. *M.A.G.*	*2*	*1*	*3*	*4*				
5. *C.L.B.*	*2*	*1*	*3*	*4*				
6. *J.W.*	*1*	*2*	*3*	*4*				
7.								
8.								

FIG. 1

designates the position of the sample in the slice. In previous tests this position in the slice remained constant or nearly so for each judge. Now, the initials of the judges are placed on the line beside the number. The initials are shifted in regular order from day to day so that each judge may have several samples from each position in the slice during the course of the experiment.

The letters A, B, C, and D denote the position of the paired slices in the roasts.

The "sample number on the plate" is determined by means of a deck of cards to prevent any bias in the order of placing which is inherent when one person tries to vary the samples deliberately. The colors form a convenient way of obtaining randomized placing for two samples—black representing the odd numbered sample on the plate and red the even numbered sample. After shuffling, the deck is cut and the top card removed and inspected. If a black card is turned up, a figure 1 is placed in the first column under A and a figure 2 in the second column. (The placing would, of course, have been reversed had a red card been obtained.) The card is replaced and this procedure repeated to give the positions of 3 and 4 under B, and if needed, of 5 and 6 under C, and of 7 and 8 under D. This completes the process for the first judge. The same method is used

for determining the position of each sample for the second judge and for each of the other judges in turn. Sometimes the sampling sheet may be filled in vertically by determining the position of the samples for each of the judges under pair A before any of those under pair B are filled in. By either of these methods, however, no judge can be certain that his sample No. 1 is like the sample No. 1 of any other judge at the table.

MEAT COOKING RECORD

JUDGING SHEET FOR TENDERNESS

Judge No. *2*

Roast numbers *667 L, R*

Date—*May 25, 1938*

Pair			A		B		C		D	
Sample No.			1	2	3	4	5	6	7	8
Paired-eating test	More tender									
	Difference	None								
		Slight		√		√				
		Decided								
Weighted adjectives	5. Very tender			√						
	4. Tender		√			√				
	3. Neutral				√					
	2. Tough									
	1. Very tough									

Signature of judge—*C.E.M.*

FIG. 2

The new judging sheet (Fig. 2) provides space for four paired judgments. The "kind of meat" and "cut of meat" formerly used are left off so that the judges may know as little as possible about their samples. The "sample number" on the judging sheet corresponds to the "sample number on the plate" found on the sampling sheet. It was determined originally by a pack of cards to insure random placing of the samples on the plate and so prevent bias in judging.

The data from the individual judging sheets (after decoding by means of the sampling sheet) is placed on the summary sheet (Fig. 3) which contains space for (1) a record of the paired judgments of each judge; (2) their grouping into differences—none, slight, and decided; and (3) the grouping of the weighted adjectives and the scores obtained from them.

As an illustration of the method, one complete set of actual data has been recorded in italic on the forms given in Figs. 1, 2, and 3.

SUMMARY SHEET

Roast numbers *667* *667*

Letters denoting position of paired slices	A		B		C		D	
Basis of pairing	*Method I*	*Method II*	*Method I*	*Method II*				
Judge	Record of individual paired judgments							
1. *A.K.M.*	*0.5*	*0.5*	*0.5*	*0.5*				
2. *C.E.M.*	*0.0*	*1.0*	*0.0*	*1.0*				
3. *P.B.P.*	*0.0*	*1.0*	*0.0*	*1.0*				
4. *M.A.G.*	*0.0*	*1.0*	*0.0*	*1.0*				
5. *C. L. B.*	*0.0*	*1.0*	*0.0*	*1.0*				
6. *J.W.*	*0.0*	*1.0*	*0.0*	*1.0*				
7.								
8.								

Roast identification		*L*		*R*	
Basis of pairing		*Method I*		*Method II*	
Difference	None	√ √	*1.0*		*1.0*
	Slight			√ √ √ √	*4.0*
	Decided			√ √ √ √ √ √	*6.0*
Total			*1.0*		*11.0*

Adjectives	Weighting	Tally of judgments	Total	Weighted	Tally of judgments	Total	Weighted
Very tender	5	√ √ √	*3*	*15*	√ √ √ √ √ √ √	*7*	*35*
Tender	4	√ √ √ √	*4*	*16*	√ √ √ √ √	*5*	*20*
Neutral	3	√ √ √ √	*4*	*12*			
Tough	2	√	*1*	*2*			
Very tough	1						
		Total	*12*	*45*		*12*	*55*
		Mean		*3.8*			*4.6*

FIG. 3

The process of recording the data is as follows: The two sets of paired slices from this pair of roasts were judged—one as Pair A and the other as Pair B. The first judge (judging sheet not given) found no difference in either pair of samples, and so on the summary sheet under "record of individual paired judgments" each sample is credited with 0.5, and two checks are placed below after "difference—none." The second judge (judging sheet given) found Sample 2 slightly more tender than Sample 1 and Sample 4 slightly more tender than Sample 3. By means of the sampling sheet, both of the tenderer samples for this judge were found to come from the roast cooked by Method II. So on the summary sheet Method II is credited with 1.0 in Pair A and 1.0 in Pair B and Method I with zero in both pairs. Two checks are placed below opposite "difference—slight" under Method II. When the data from all of the individual judging sheets are recorded, it is found from the totals that out of 12 paired judgments, 11 of them were in favor of the roast cooked by Method II.

While weighted adjectives are given on the judging sheet and summary sheet, they are used only for the purpose of forming an idea whether we are dealing with tender meat or tough meat. The scores are not part of the paired-eating method and are not used in interpreting the data. The scoring by means of adjectives, however, appears to be more definitely comparative when only two samples are used. The scores from the weighted adjectives for these roasts were 4.6 for that cooked by Method II and 3.8 for that cooked by Method I.

This random method of placing the samples obviously prevents bias in the judgments but adds considerably to the work of preparing the samples and recording the data.

STATISTICAL ANALYSIS

The simplicity of the statistical analysis is one of the important advantages of the paired-eating method. Statistical treatment of the data may be made either by the binomial method or by the method of Chi-square. Since there is only one degree of freedom, the interpretation of results should be the same whichever method is selected. As the binomial method has been given in the previous publication, the method using Chi-square will be described here.

The paired-eating method is concerned with the differences in N observations on the effect of two treatments on paired roasts. Quantitative data are lacking, so the differences are recorded as +1 under the treatment preferred and as zero under the other. If no difference is observed +0.5 is recorded under each treatment. Use is made of the following symbols and terms:

X_1 = number recorded under one treatment (the more favored one in the final total).
X_2 = number recorded under the other treatment.
$N = X_1 + X_2$ = total number of observations (paired judgments).

$$\text{Tenderness-percentage} = \frac{X_1}{N} \times 100.$$

$$\text{Chi-square} = \frac{(X_1 - X_2)^2}{N}$$ (Special formula, convenient when testing a 50:50 hypothesis).

A Chi-square below 3.841 is not significant; above 6.635 is usually considered highly significant for one degree of freedom as here.

DISCUSSION

In the paired-eating method the judge has a chance to record small differences if he detects them because he is comparing or contrasting only two samples. Differences which are too small to be real will likely be recorded in favor of one roast one time and in favor of the other roast another time and will, therefore, average out as if they had been guesses. Where the judgments are largely of this type, a tenderness-percentage approaching 50 is expected. (Results owing to chance alone are shown by a 50:50 ratio.) Where the difference is real, the data will differ significantly from the 50:50 ratio.

That the paired-eating method is well adapted to detecting real differences is illustrated in the following comparison; there was no

TABLE 1

Summary of Data by Individual Judges for Tenderness of Paired Chuck Roasts Cooked Alike [1]

Judge	Number of paired roasts	Number of paired judgments		Tenderness-percentage $X_1/N \times 100$	Chi-square [2]
		Total N	More tender, left, X_1		
J.W.	10	23	14.0	61	1.1
M.A.G.	8	15	5.0	33	1.7
C.E.M.	11	23	12.0	52	0.04
P.B.P.	11	23	10.5	46	0.03
A.K.M.	11	23	9.5	41	0.7
C.L.B.	8	15	9.0	60	1.7
All judges	11	122	60.0	49	0.03

[1] Temperatures—oven 125°C.(257°F.); internal 80°C.(176°F.). [2] A Chi-square below 3.841 is not significant.

detectable difference in tenderness between left and right arm-bone chuck roasts when paired and cooked well-done under the same oven conditions, but when the oven conditions were changed to test the effect of high and low oven temperature, there was a highly significant difference in tenderness in favor of the low oven temperature. The detailed data bring this out clearly.

The data from the chuck roasts cooked alike (Table 1) show that the tenderness-percentage for all judges is 49 with a Chi-square of .03. The difference from the expected 50:50 ratio is not significant

TABLE 2

Individual Judgments for Tenderness of Individual Left Chuck Roasts Cooked the Same as Their Right Pair Mates

Roast No.	J.W.	M.A.G.	C.E.M.	P.B.P.	A.K.M.	C.L.B.	X_1	N
663L	0.5	0.0	0.5	0.5	0.0	1.0	2.5	6
	1.0	0.0	1.0	1.0	1.0	0.0		
665L	0.0	1.0	0.0	0.0	0.0	0.0	5.0	12
	0.5	0.0	0.5	0.0	0.5	0.5		
669L	0.5	0.0	0.5	0.5	1.0	0.5	5.0	12
	0.5	1.0	0.5	0.5	1.0	0.5		
670L	1.0	1.0	1.0	0.5	0.0	0.5	8.0	12
	0.5	0.0	0.5	0.5	0.0			
673L	0.5	0.5	0.5	0.5	1.0		4.5	10
	1.0	0.5	0.5	0.5	0.0			
674L	1.0	0.5	0.5	0.5	0.0		5.0	10
	0.0	0.0	0.0	0.5	0.5	0.5		
675L	0.5	0.0	0.5	0.5	0.5	0.5	4.0	12
			0.5	0.0	0.5			
			0.5	0.5	0.5			
657L			0.5	0.5	0.5		5.5	12
			0.5	0.5	0.5			
	0.5	0.5	0.5	0.5	0.0	1.0		
688L	0.5	0.0	0.5	0.0	0.0	1.0	5.0	12
	1.0							
	1.0							
	0.5		0.5	0.5	0.0	1.0		
697L	1.0		0.5	1.0	1.0	1.0	9.0	12
	0.5							
	1.0							
	0.0		0.5	0.5	0.5	0.5		
698L	0.5		1.0	0.5	0.5	0.5	6.5	12
Total	14.0	5.0	12.0	10.5	9.5	9.0	60.0	122
Total possible	23.0	15.0	23.0	23.0	23.0	15.0	122.0	122

and the data may be interpreted as showing no difference in tenderness between paired arm-bone chuck roasts when cooked alike. This finding is in line with our working hypothesis that a left roast when paired with a right roast of the corresponding cut is similar in tenderness. Notice also that the Chi-square for *none* of the judges is high enough to be significant, showing that none of the judges was able to detect a significant difference between the paired roasts. The record of each of these judges for each roast (the records from which the data in Table 1 were compiled) are presented (Table 2). Notice that there were many times when the judges reported differences but that these differences were in favor of one roast one time and in favor of the other roast another time and so averaged out as if they had been guesses. If these differences in tenderness were real, the samples from which they were obtained were distributed in a random manner in both left and right roasts and so did not influence the final results.

The data for the high and low oven temperatures (Table 3) show

TABLE 3

Summary of Data by Individual Judges for Tenderness of Well-Done Chuck Roasts Cooked at Oven Temperatures 125 and 225°C.(257 and 437°F.)

Judge	Number of paired roasts	Number of paired judgments		Tenderness-percentage $X_1/N \times 100$	Chi-square [1]
		Total N	More tender, 125°C., X_1		
J.W.	17	35	33.5	96	29
M.A.G.	16	33	32.0	97	29
J.H.K.	13	26	25.0	96	22
D.S.B.	11	22	22.0	100	22
A.K.M.	13	27	26.0	96	23
J.S.	1	3	2.0	66	
J.M.J.	1	2	2.0	100	
All judges	17	148	142.5	96	127

[1] A Chi-square above 6.635 is highly significant.

that the tenderness-percentage for the total of all judges is 96—almost unanimously in favor of the low oven temperature method. The Chi-square of 127 indicates a highly significant difference from the 50:50 ratio and shows that the results are not due to chance. The Chi-square for each judge who tested a large enough number of samples (J.S. and J.M.J. did not) is also large enough to be highly significant, showing that each judge was able to detect a real difference in tenderness between the paired roasts. The record of each of these judges for each roast (the records from which the data in Table 3 were compiled) is presented (Table 4). Notice that in this

TABLE 4

Individual Judgments for Tenderness of Individual Pairs of Well-Done Chuck Roasts Cooked at 125°C.(257°F.)

Carcass grade	Roast No.	J.W.	M.A.G.	J.H.K.	D.S.B.	A.K.M.	J.S.	J.M.J.	X_1	N
		1.0		1.0	1.0	1.0				
Prime	231L	1.0		1.0	1.0	1.0			8.0	8
		1.0	1.0			1.0	0.0			
		0.0	0.0	1.0		0.0	1.0			
Choice	52R	1.0	1.0	1.0		1.0	1.0		10.0	14
		1.0	1.0	1.0	1.0					
	80R	1.0	1.0	1.0	1.0				8.0	8
		1.0	1.0	1.0	1.0					
	92R	1.0	1.0	1.0	1.0				8.0	8
		1.0	1.0	1.0	1.0			1.0		
	70R	1.0	1.0	1.0	1.0			1.0	10.0	10
		1.0	1.0	1.0	1.0					
Good	180R	1.0	1.0	1.0	1.0				8.0	8
		1.0	1.0	1.0		1.0				
	181L	1.0	1.0	1.0		1.0			8.0	8
		1.0	1.0	1.0	1.0	1.0				
Medium	195L	1.0	1.0	1.0	1.0	1.0			10.0	10
		1.0	1.0	1.0	1.0	1.0				
	198R	1.0	1.0	1.0	1.0	1.0			10.0	10
		0.5	1.0	0.5		1.0				
	171L	1.0	1.0	0.5		1.0			6.5	8
		1.0	1.0	1.0		1.0				
	174R	1.0	1.0	1.0		1.0			8.0	8
		1.0	1.0		1.0	1.0				
Common	187L	1.0	1.0		1.0	1.0			8.0	8
		1.0	1.0		1.0	1.0				
	190R	1.0	1.0		1.0	1.0			8.0	8
		1.0	1.0	1.0	1.0	1.0				
	207L	1.0	1.0	1.0	1.0	1.0			10.0	10
		1.0	1.0	1.0	1.0	1.0				
	210R	1.0	1.0	1.0	1.0	1.0			10.0	10
		1.0	1.0			1.0				
	215L	1.0	1.0			1.0			6.0	6
		1.0	1.0			1.0				
	218R	1.0	1.0			1.0			6.0	6
Total.....		33.5	32.0	25.0	22.0	26.0	2.0	2.0	142.5	148
Total possible.....		35.0	33.0	26.0	22.0	27.0	3.0	2.0	148.0	148

TABLE 5

Tenderness of Individual Pairs of Well-Done Chuck Roasts

Carcass		Ripening period	Roast No.		Number of judgments by paired-eating method			
					Total N	For statistical treatment		
						More tender		Percentage $X_1/N \times 100$
U.S. grade	Weight		125°C. (257°F.)	225°C. (437°F.)		125°C. X_1	225°C. X_2	
	lb.	*days*						
Prime	600-700	10	231L	232R	8	8.0	0.0	100
Choice	500-600	7	52R	51L	14	10.0	4.0	
		14	80R	79L	8	8.0	0.0	
		6	92R	91L	8	8.0	0.0	
		Total	.....	.....	30	26.0	4.0	87
	400-500	6	70R	69L	10	10.0	0.0	100
Good	500-600	9	180R	179L	8	8.0	0.0	
		9	181L	182R	8	8.0	0.0	
		Total	.....	.....	16	16.0	0.0	100
Medium	500-600	9	195L	196R	10	10.0	0.0	
		9	198R	197L	10	10.0	0.0	
		Total	.....	.....	20	20.0	0.0	100
	300-400	10	171L	172R	8	6.5	1.5	
		10	174R	173L	8	8.0	0.0	
		Total	.....	.....	16	14.5	1.5	91
Common	500-600	9	187L	188R	8	8.0	0.0	
		9	190R	189L	8	8.0	0.0	
		9	207L	208R	10	10.0	0.0	
		9	210R	209L	10	10.0	0.0	
		9	215L	216R	6	6.0	0.0	
		9	218R	217L	6	6.0	0.0	
		Total	.....	.....	48	48.0	0.0	100
Total for all roasts					148	142.5	5.5	96

case the differences reported are quite uniformly in favor of the low oven temperature. Similar uniformity is observed in the tenderness-percentages when these data are summarized by grade and weight of carcass (Table 5). This consistency of results when logical but different groupings are made of the data is a further test of significance.

Since the same cut of meat was used in both tests and since the total number of judgments in Tables 1 and 3 are approximately the same, a finding of no difference in one case and of such a highly significant difference in the other may be interpreted as showing that the method is capable of detecting real differences.

The selection of judges at most of the Experiment Stations must, of necessity, be made from the limited number of persons who are willing to serve and who are able to attend fairly regularly. No

method has yet been devised for detecting persons who will make superior judges for using the paired-eating method. From the results given (Tables 1 and 3) all of the judges used were quite satisfactory and apparently equally so if they judged a sufficient number of samples to permit a valid statistical analysis of the results. A preliminary period, however, is desirable for training each judge in the mechanics of the method. After this preliminary period anyone of average intelligence and with average ability to concentrate ought to make a good judge. The judging committee should consist of more than two persons because of the large number of samples to be tested (see next paragraph), but the upper limit will be set either by the limited number of persons available regularly or by the amount of meat available for judging.

The problem of sampling is one of the most trying in meat research. Variations in amount and distribution of fat deposits within a muscle and the amount, character, and distribution of connective tissue vary greatly even from slice to slice in the same roast and from sample to sample in the same slice. Errors in sampling are minimized in two ways with the paired-eating method: First, in each slice practically the entire area of the muscle which is free from coarse connective tissue is used for sampling. This amount of meat is divided into several individual samples for each of which paired judgments are recorded. Since meat is not homogenous in the state in which it is desirable to test it, a large number of individual observations of the entire area of at least two slices is a decided advantage. Second, quantitative values are not used and so it is impossible for one exceedingly tough sample to overbalance several others which are more tender. For instance, suppose one sample from the left roast were extremely tough—much tougher than its pair-mate from the right roast. Suppose, also, that the other eight samples from this slice of the eye muscle were slightly more tender than their pair-mates from the right roast. With the paired-eating method the one tougher sample—no matter how much tougher—would count as only one sample and could not influence the results more than its share of the total number of samples. Had quantitative values been used, there would have been no way to keep this unusual sample from outweighting several others of the same area.

Gradations in tenderness-response are often an advantage for they may reveal clues to a better interpretation of the results than would have been possible without them. Such gradations may be obtained by the paired-eating method when several additional variables are present, provided sufficient well-planned data have been

TABLE 6

Gradations in Tenderness-Response of Paired Roasts to High and Low Oven Temperatures

Stage of cooking	Cut	Number of paired roasts	Number of paired judgments		Tenderness-percentage $X_1/N \times 100$	Chi-square [1]
			Total N	125°C. (257°F.) X_1		
Well-done	Chuck	17	148	142.5	96	126
Well-done	Rib	26	263	182.5	69	40
Difference	.....	.....	.....	.....	27	41 [2]
Medium-rare	Rib	22	263	142.0	54	1.7
Medium-rare	Chuck	21	178	95.5	54	1.0
Difference	.....	.....	.....	.....	0	.....
Well-done	Chuck	17	148	142.5	96	126
Medium-rare	Chuck	21	178	95.5	54	1.0
Difference	.....	.....	.....	.....	42	75
Well-done	Rib	26	263	182.5	69	40
Medium-rare	Rib	22	263	142.0	54	1.7
Difference	.....	.....	.....	.....	15	13

[1] A Chi-square above 6.635 is highly significant, below 3.841 is not significant.

[2] $\text{Chi-square} = \text{Sum} \dfrac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$ which applied to these data becomes

$$\frac{(142.5 - 117.03)^2}{117.03} + \frac{(5.5 - 30.97)^2}{30.97} + \frac{(182.5 - 207.97)^2}{207.97} + \frac{(80.5 - 55.03)^2}{55.03} = 41$$

The "expected" values for the above equation are obtained as follows: Assume that the "observed" total (325) under 125°C. in the table below is the true proportion of the grand total (411) for the rib and chuck separately as well as together. (It would be if there were no difference in the responses of chuck and rib to oven temperature.) Out of the total judgments of the chuck (148), the correct proportion ("expected") would then be 117.03 for 125°C.(257°F.) and 30.97 for 225°C.(437°F.) calculated as follows:

$$\text{Expected (Chuck, 125°C.)} = \frac{325}{411} \times 148 = 117.03$$

$$\text{Expected (Chuck, 225°C.)} = 148 - 117.03 = 30.97$$
$$\text{Expected (Rib, 125°C.)} = 325 - 117.03 = 207.97$$
$$\text{Expected (Rib, 225°C.)} = 263 - 207.97 = 55.03$$

Roasts compared		125°C.	225°C.	Total
Observed	Well-done chuck	142.5	5.5	148
	Well-done rib	182.5	80.5	263
	Total	325.0	86.0	411
Expected	Well-done chuck	117.03	30.97	148
	Well-done rib	207.97	55.03	263

collected. It is important, however, that the gradations be gradations in *tenderness-response to a common independent variable*, not just gradations in the *tenderness* of individual samples. In other words, paired samples must be used but the different pairs may represent different cuts or different conditions in the testing of the same variable; an illustration is given (Table 6). All of the paired judgments

were made with one independent variable—oven temperature. But these observations were made on two cuts and at two stages of doneness for each cut. This permitted four comparisons to show gradations in tenderness-response.

The first comparison (Table 6) is between the two cuts when both were cooked well-done. The difference in tenderness-percentage was 27. The Chi-square of 41 (highly significant) shows that the two oven temperatures produced a difference in the tenderness of well-done meat which was more pronounced in the chuck roasts than in the rib roasts. The reason for this difference in tenderness-response of the two cuts was not apparent at that time.

In the second comparison, the response in tenderness to high and low oven temperatures was observed on the same cuts cooked medium-rare. It is remarkable that there is no difference in tenderness-percentage when the same cuts were subjected to conditions which were similar to those in the preceding test except that the stage of cooking was medium-rare instead of well-done.

In the third and fourth comparisons (Table 6), the data were arranged so that medium and well-done roasts of the same cut were compared for their response to high and low oven temperatures. The difference in tenderness-response between the well-done and medium-rare chuck roasts was 43 with a Chi-square of 75. The difference between the well-done and medium-rare rib roasts was 15 with a Chi-square of 13. Both differences are highly significant. The tendering effect of the low oven temperature (or the toughening effect of the high oven temperature) was, therefore, much greater in the well-done roasts than in the medium-rare ones.

Whatever may have been causing a tenderness-response to extremes in oven temperature, it seems clear that in these tests it was operating only between the medium-rare and the well-done stages of cooking and that it was more effective in the well-done chuck roasts than in the well-done rib roasts.

The description and discussion of the paired-eating method have been limited to its use in testing tenderness in meat since it has been used only for this purpose in this laboratory. There seems to be no reason why any other factor of palatability in meat or other foods may not be tested by this method if suitable words are substituted in the forms. But it should be emphasized that the paired-eating method is applicable only to those cases in which the difference between two comparable samples is the important consideration. It cannot be used for comparing a large number of individual samples with each other, nor for comparing individual samples cooked on different days.

SUMMARY

The paired-eating method is presented as a method designed to overcome certain of the difficulties encountered in studying the nature and causes of those changes taking place in the cooking process which influence the palatability of food. Its use is illustrated by tests for tenderness in meat.

It is applicable to those studies in which the major interest is concentrated on observing the effect of the extremes of one independent variable on one factor of palatability as the dependent variable.

Its essential and distinctive features are the providing of paired samples and the judging of the paired samples by comparison with each other.

The statistical analysis is simple and the calculations require only a short period of time.

ACKNOWLEDGMENT

Grateful acknowledgment is made to Dr. A. E. Brandt, Senior Statistician, Soils Conservation Service, Washington, D. C., for his suggestions in regard to sampling and to Mr. C. B. Godbey of the Genetics Department of A. & M. College for his suggestions regarding appropriate statistical methods for analyzing the data.

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RIBOFLAVIN CONTENT OF SOME COMMON VEGETABLES AND FRUITS

A. Z. HODSON
Department of Poultry Husbandry, Cornell University, Ithaca, New York

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The fact that riboflavin is required for growth in many species of animals has been considered for a long time sufficient evidence of the need for this vitamin in the human dietary. Sebrell and Butler (1938) reported that they had produced experimentally a clinical syndrome of riboflavin deficiency in human beings in which cheilosis (lesions on the lips in the angles of the mouth) is one of the symptoms.

Oden, Oden, and Sebrell (1939) encountered cases of this deficiency in Georgia and successfully treated them with synthetic riboflavin. The latter authors state that in all probability it is a common dietary deficiency in southern United States. Vilter, Vilter, and Spies (1939) also noted cases of riboflavin deficiency in human beings. Spies, Bean, and Ashe (1939) report a rather extensive study of riboflavin, thiamin, and nicotinic acid deficiencies. These reports and others suggest the need for further quantitative determinations of the riboflavin content of our more common foods, particularly for vegetables and fruits.

Daniel and Munsell (1937), in their survey of the literature, included quantitative values for vitamin G in 25 fruits and vegetables in their digest of quantitative values, although values for about 40 were presented in their more comprehensive tables. McCollum, Orent-Keiles, and Day (1939) include values for the riboflavin content of only 12 vegetables and fruits in their book, "The Newer Knowledge of Nutrition." As is pointed out by these authors on page 490, many of the data obtained by the rat-growth method are measures of the vitamin G complex rather than of riboflavin. This is especially true of the work prior to 1932, since before that time no attempt was made to use a standard procedure.

The development of microbiological methods, by Snell and Strong (1939), and chemical methods, by Hodson and Norris (1939), which are more rapid and more specific, have made it possible to extend the existing knowledge on the riboflavin content of vegetables and fruits.

EXPERIMENTAL PROCEDURE

The samples of vegetables and fruits were selected on the local market. Care was taken to select edible samples which were repre-

sentative. No spoiled or wilted samples were included. Since fresh asparagus was not available, a commercial sample of frozen asparagus was obtained.

Unless otherwise noted, only the edible portion of the vegetable or fruit was used. In most cases the skins of fruits and of roots

TABLE 1

Riboflavin Content of Fresh Vegetables and Fruits

Sample No.	Vegetable or fruit	Dry matter *pct.*	Micrograms of riboflavin per gram	
			Dry matter	Fresh weight
292	Apples	14.6	5.0	0.73
289	Asparagus (frozen)	7.8	16.6	1.29
291	Bananas	22.2	3.8	0.84
268	String beans (green)	8.3	11.2	0.93
265	Wax beans (yellow)	8.9	11.3	1.01
251	Beets	15.3	1.5	0.23
249	Beet tops	10.0	8.2	0.82
266	Broccoli	12.5	16.8	2.10
280	Broccoli (overmature)	20.2	10.6	2.14
288	Broccoli	15.2	14.9	2.26
260	Cabbage	9.3	7.1	0.66
277	Cabbage	7.6	5.8	0.44
271	Chinese cabbage	4.6	8.3	0.38
298	Cantaloupe	8.8	8.3	0.73
253	Carrots	13.7	4.9	0.67
276	Cauliflower	9.0	14.1	1.27
255	Celery	5.8	6.0	0.35
250	Swiss chard	9.2	15.0	1.38
256	Swiss chard stems	5.9	3.5	0.21
261	Sweet corn (maize) (roasting-ear stage)	30.9	3.9	1.21
283	Sweet corn (maize) (mature roasting-ear stage)	37.6	1.9	0.71
275	Cucumber	4.1	10.8	0.44
272	Eggplant	7.1	4.4	0.31
270	Endive	4.4	13.8	0.61
295	Red grapes	18.8	1.7	0.32
296	White grapes	20.3	4.5	0.92
279	Head lettuce	3.6	13.4	0.48
248	Leaf lettuce (stemmy)	10.2	9.4	0.96
278	Leaf lettuce	4.9	14.6	0.71
281	Onions	13.0	9.5	1.23
294	Peaches	15.6	4.1	0.64
293	Pears	13.9	5.5	0.76
264	Peas	21.4	7.2	1.54
284	Peas	23.4	5.6	1.31
282	Green peppers	8.2	16.8	1.38
257	Potatoes	20.5	2.7	0.55
287	Potatoes	20.9	2.1	0.44
258	Sweet potatoes	33.1	2.1	0.70
286	Sweet potatoes	34.3	2.0	0.68
285	Radishes	5.1	6.8	0.35
267	Romaine	9.6	10.0	0.96
259	Spinach	7.1	22.5	1.60
269	Spinach	7.8	20.4	1.59
273	Hubbard squash	8.1	5.7	0.46
262	Summer squash	5.4	9.6	0.52
254	Tomatoes	6.7	7.8	0.52
274	Turnips	9.5	4.4	0.42

were retained as part of the sample. Potatoes, sweet potatoes, and turnips, however, were pared.

In preparing the samples for analysis they were cut in small pieces, or in some cases grated, and then dried in a hot air tunnel at 60°C.(140°F.) until the samples were reasonably dry. The last traces of moisture were removed by placing the samples in an oven at 60°C. with 24 inches of vacuum for four hours. The drying periods were doubled for fruits, since they were more difficult to dry. No further loss in weight was noted on repeating the oven drying on a few representative samples of vegetables and of fruits. Since riboflavin is relatively stable to heat and to oxidation, no destruction of it results when the samples are dried under the stated conditions.

The riboflavin was determined by the fluorometric method of Hodson and Norris (1939). The samples were protected from light as much as possible. The manipulation of the extracts was carried on in red light. The results of the investigation are presented (Table 1).

DISCUSSION

Since this investigation is of a preliminary nature, an attempt has been made to study a number of different vegetables, rather than to establish an accurate average value for any one vegetable. In some cases the riboflavin content of two or more samples of a vegetable was determined. Although considerable variation was shown, in no case was the difference wide. A further, more comprehensive study of the effect of environmental conditions and of varietal differences will no doubt be necessary before a definite average value can be assigned for the riboflavin content of any vegetable or fruit. Nevertheless, in the absence of more complete data, this preliminary study offers considerable information on the riboflavin content of some of our more common vegetables and fruits.

The data indicate that when the vegetables and fruits are considered on the basis of riboflavin content per unit of dry matter, they may be roughly divided into three classes: those containing 10 to 20, those containing 5 to 9.9, and those containing 0 to 4.9 micrograms of riboflavin per gram.

On the dry basis, the best vegetable sources of riboflavin are the green vegetables, such as broccoli, spinach, lettuce, Swiss chard, endive, asparagus, etc. They supply more than 10 micrograms of riboflavin per gram of dry matter.

Included in the class which supplies 5 to 9.9 micrograms of riboflavin per gram are the white vegetables, cabbage, Chinese cabbage, and celery; some of the vegetable fruits, such as tomato and squash; and some of the fruits, such as apple and pear. Included

in the class of vegetables which supplies less than 4.9 micrograms of riboflavin are the roots and tubers, potatoes, sweet potatoes, beets, carrots, etc. Sweet corn, eggplant, and a few of the fruits also fall in this class. No logical grouping of the vegetables or fruits according to their riboflavin content on a fresh basis was found.

The fact that the green vegetables are good sources of riboflavin should prove useful in reducing the incidence of riboflavin deficiency. This is especially true in areas where green vegetables are readily available, but where supplies of milk, meat, and eggs are limited and hence are relatively expensive.

SUMMARY

A preliminary investigation of the riboflavin content of some common fruits and vegetables has been made.

The vegetables have been roughly grouped into three classes according to their riboflavin content per unit of dry matter.

The green vegetables, such as spinach, lettuce, broccoli, and Swiss chard, proved to be the best vegetable sources of riboflavin, especially when the riboflavin content is considered on the basis of dry weight.

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THERMAL PROCESSING OF CANNED FOODS IN TIN CONTAINERS

III. RECENT IMPROVEMENTS IN THE GENERAL METHOD OF THERMAL PROCESS CALCULATIONS — A SPECIAL COÖRDINATE PAPER AND METHODS OF CONVERTING INITIAL AND RETORT TEMPERATURES

O. T. SCHULTZ[1] AND F. C. W. OLSON
Research Department, American Can Company, Maywood, Illinois

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The first method developed for making calculations of processing times for canned foods—the General Method—has been described in a classic paper by Bigelow, Bohart, Richardson, and Ball (1920). This method, which is still used in the canning industry, is especially valuable when the heating curve for the product under consideration cannot be represented by one or two straight lines on semi-logarithmic paper. If the heating curves can be so represented, the process calculations may be made by the simpler and more versatile methods devised by Ball (1923, 1928).

One of the requirements of the General Method is that the thermal death times of the organism being destroyed by the process must be known at all temperatures attained during the process. Practically, this knowledge is obtained by determining the thermal death times at several temperatures in the processing range. The data so obtained are plotted using a logarithmic time scale and a linear temperature scale. The resulting points are connected by a smooth curve from which thermal death times are found for all can temperatures obtained during the process. It is not necessary to know the mathematical formula relating the thermal death time with the temperature.

PROCESS CALCULATION BY GENERAL METHOD

The General Method for process calculation can best be described by solution of a definite problem. Assume that a heat-penetration test has been made (Columns 1 and 2, Table 2), and assume further that the thermal death times of the organism to be destroyed by this process are known for all temperatures in the processing range (Fig. 2). If the heat-penetration data are plotted on semi-logarithmic paper (Fig. 1), it will be difficult to represent the heating curve by one or two straight lines. Furthermore, the slope of the cooling curve is not

[1] Now associated with U. S. Naval Observatory, Washington, D. C.

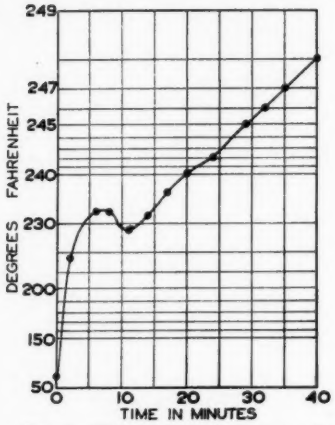

FIG. 1. Heat-penetration curve plotted on semi-logarithmic paper.

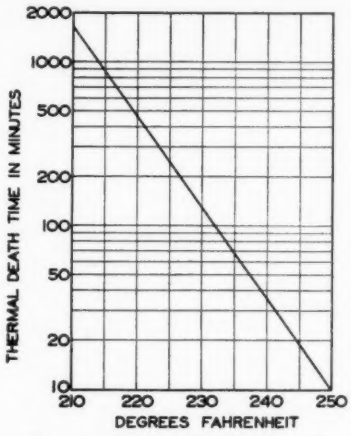

FIG. 2. Thermal death-time curve of organism to be destroyed by thermal process.

at all comparable to the slope of the heating curve. In a case such as this, the formula methods developed by Ball (1923, 1928) are not readily applicable and the General Method must be used. Knowing the time-temperature relations in the can and the lethal rate of the

TABLE 2

Data Used in Plotting Lethality Curve

Time	Temperature	Thermal death time	Lethal rate
min.	°F.		
0	82	.....	
2	217	670	.0015
4	230	129	.0078
6	233	88	.0114
8	233	88	.0114
11	228	165	.0061
14	232	100	.0100
17	237	53	.0189
20	240	36	.0278
24	242	28	.0357
29	245	19	.0526
32	246	16.7	.0599
35	247	14.8	.0676
40	248	13	.0769
45	248.5	12.4	.0806
47	248.5	12.4	.0806
47.5	247	14.8	.0676
48	230	129	.0078
49	217	670	.0015

organism to be destroyed, the length of time must be found which is just sufficient to destroy the organism.

The problem is solved systematically by entering the heat-penetration data in Columns 1 and 2 (Table 2). The thermal death times corresponding to the temperatures in Column 2 are obtained from Fig. 2 and entered in Column 3. The lethal rates in Column 4 are found by taking the reciprocals of the thermal death times in Column 3. The data in Columns 1 and 4 are then plotted on cross-section paper (Fig. 3), and the points are joined by a smooth curve to form a "lethality curve."

A "unit-sterilization area" is defined as that area on a lethality diagram which just represents complete sterilization. The size of

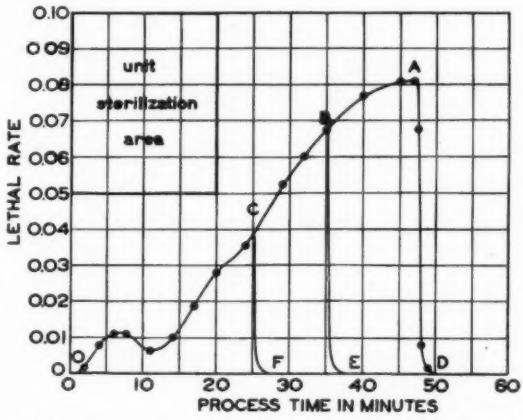

FIG. 3. Lethality curve plotted from Columns 1 and 4, Table 2.

this area depends on the scales chosen for the lethality diagram. To find the size, a rectangle is constructed on the lethality diagram of such size that the product of its height and width is unity. In Fig. 3, for example, the unit-sterilization area is a rectangle having a height of .05 along the lethal-rate scale and a width of 20 minutes along the time scale; the area is then $.05 \times 20 = 1$.

The area OCBADEFO under the lethality curve representing a 47-minute process is found to be 192 per cent of the unit-sterilization area. This means that the process is considerably longer than necessary. To find the time required for unit sterilization under the lethality curve, cooling curves similar to the original cooling curve are drawn from several places along the heating curve. Two cooling curves are drawn (Fig. 3), starting at 25 and 35 minutes. The areas

under these curves (OCFO and OCBEFO) are 42 and 97 per cent of the unit-sterilization area. By graphical interpolation, it is found that after processing 35.5 minutes complete sterilization is obtained.

It is readily seen that this method is laborious and not well suited for routine calculations. Ball (1928) made several noteworthy improvements on the General Method, the chief of which was assuming a straight-line, thermal death-time curve passing through the point one minute at 121.1°C.(250°F.) (F = 1). The area under the lethality curve in terms of the unit-sterilizing area will then give the sterilizing value (F value) of the process directly. Another improvement consisted of calculating the lethal rate from the equation

$$L = \log^{-1} \frac{T - 250}{z}$$

where T is the temperature in degrees Fahrenheit and z is the slope of the thermal death-time curve. Double-entry tables can then be prepared giving the lethal rate for any temperature and z value; an example of such a table is given (Table 1).

TABLE 1

Lethal Rates for Various Temperatures and Values of z

Temperature	z = 10	z = 14	z = 18	z = 22	z = 26
°F.					
216	0.0004	0.0037	0.0129	0.0285	0.0492
218	0.0006	0.0052	0.0167	0.0351	0.0588
220	0.0010	0.0072	0.0215	0.0433	0.0702
222	0.0016	0.0100	0.0278	0.0534	0.0838
224	0.0025	0.0139	0.0359	0.0658	0.1000
226	0.0040	0.0193	0.0464	0.0811	0.1194
228	0.0063	0.0268	0.0599	0.1000	0.1425
230	0.0100	0.0374	0.0774	0.1233	0.1701
232	0.0159	0.0518	0.1000	0.1520	0.2031
234	0.0252	0.0721	0.1292	0.1874	0.2425
236	0.0398	0.1000	0.1668	0.2310	0.2894
238	0.0631	0.1390	0.2154	0.2848	0.3455
240	0.1000	0.1931	0.2783	0.3512	0.4125
242	0.1585	0.2683	0.3594	0.4329	0.4924
244	0.2522	0.3737	0.4642	0.5336	0.5878
246	0.3981	0.5179	0.5993	0.6579	0.7017
248	0.6310	0.7210	0.7743	0.8111	0.8377
250	1.0000	1.0000	1.0000	1.0000	1.0000
252	1.585	1.390	1.292	1.233	1.194
254	2.522	1.931	1.668	1.520	1.425
256	3.981	2.683	2.154	1.874	1.701
258	6.310	3.737	2.783	2.310	2.031
260	10.000	5.179	3.594	2.848	2.424

CONSTRUCTION OF SPECIAL COÖRDINATE OR LETHAL-RATE PAPER

The special coördinate paper about to be described represents a further improvement in the General Method in that calculations may be made with considerably less effort and the chances of misplotting points are decreased. For clarity of exposition, a z value of 18 is assumed in the description of the construction of this special coördinate paper. The application to other values of z is obvious.

A sheet of paper is ruled with two horizontal lines unit distance apart. The bottom line will be referred to as the zero line and the top line is marked 250°F. for the present. For every degree of temperature in the processing range, (for the present, it is assumed this does not exceed 250°F.) the value of L is calculated (or found in Table 1) and a horizontal line is drawn whose distance is L units above the zero line. The lines are numbered not with the values of L but with the corresponding values of T. Vertical lines for the time scale may be ruled in any convenient manner provided that the time scale is linear. A paper ruled in this manner is illustrated (Fig. 4).

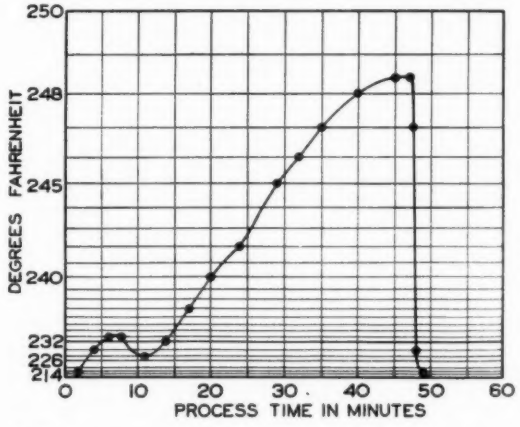

FIG. 4. Lethality curve plotted on lethal rate paper directly from heat-penetration data.

The objection might properly be raised that this construction allows no choice in the units for the vertical scale. For instance, if the highest temperature reached in the heat-penetration test were 227°F., the curve would occupy a very narrow band at the bottom of the page and would be too small to be measured conveniently with a planimeter. This difficulty is not so serious as might be thought at first, as the following considerations will show.

Consider two temperatures T and T′ such that

$$T - 18 = T'.$$

Then, when T — 250 is divided by 18, the remainder is the same as when T′ — 250 is divided by 18. Since the remainder is used as the mantissa of a common logarithm, the antilog of (T — 250)/18 will be represented by the same sequence of digits as the antilog of (T′ — 250)/18 and the antilogs will differ only by a factor of 10. The 18 integers from 250 to 233 have values of L each 10 times as great as the values of L corresponding to the 18 integers from 232 to 215. An extension of this argument will show that the values of L run in cycles corresponding to an 18°F. change in T, and the values of L in any cycle differ from the values in the cycle between 250 and 233 only by some factor of 10. Hence, instead of numbering the top line 250, it could be numbered 268, 250, 232, 214, etc. This allows a certain freedom in the choice of the vertical scale. If, as before, the highest temperature reached were 227°F. the top line could be numbered 232 and then the curve would extend over half the page. Thus, by a proper choice of scale, it is always possible to have the top of the curve somewhere in the upper nine-tenths of the page.

At the laboratories of the American Can Company, where a great number of calculations have to be made as an almost routine matter, it was found desirable to have one of the large graph paper manufacturers make a special plate for printing lethal-rate paper based on z = 18, which is the most commonly used value of z. For other values of z, a sheet is carefully ruled and copies made by the planograph process.

When doing very accurate work, it is necessary to check the size of the paper to determine possible shrinkage and make the proper corrections.

USE OF LETHAL-RATE PAPER IN PROCESS CALCULATIONS

In using this special coördinate paper, points are marked on it corresponding to the time-temperature points of the heat-penetration data. A smooth curve is drawn connecting the points, and the area under the curve is measured by any convenient means, preferably a planimeter. The F value of the process is obtained from the area under the curve by the formula

$$F = \frac{mA}{10^{n}d}$$

where m = number of minutes represented by one inch on the time scale
A = area under the curve in square inches
n = the number of 18°F. changes that must be made to change the number of the top line from 250 to the desired number (n is positive if the top line is numbered less than 250 and negative if it is numbered greater than 250)
d = number of inches from the zero line to the top line.

Finding the area under the curve and then the F value permits one to evaluate or determine the sterilizing value of a process. If it is desired to calculate a process having a given F value, cooling curves are drawn as in the General Method described above. The areas under these curves are found and the F values obtained from the above formula. The F values so obtained are plotted against the corresponding process times. By drawing a curve through these points, the length of process having the desired F value is easily found.

CONVERSION OF RETORT AND INITIAL TEMPERATURES IN GENERAL METHOD CALCULATIONS

If an experimental heat-penetration curve has been determined for a particular retort temperature, and it is desired to find the corresponding can temperatures for different retort and initial temperatures, the conversions may be made by the following methods:

When the initial temperature remains the same but the retort temperature is changed,

$$CT' = RT' - \frac{RT' - IT}{RT - IT}(RT - CT)$$

where RT = original retort temperature
RT′ = new retort temperature
IT = initial temperature
CT = a can temperature of the original set
CT′ = a new can temperature corresponding to CT.

If CT′ is plotted against CT, the graph will be a straight line since CT′ is a linear function of CT. Hence, it is necessary to use the formula for only two temperatures and join the points with a straight line. Any two values of CT may be selected for this purpose. If CT = RT, then CT′ = RT′ and hence one point on the line will be that represented by the two retort temperatures. Similarly, if CT = IT, then CT′ = IT, and hence another point will be that represented

by the two (equal) initial temperatures. By using these two points it is possible to draw the line without using the formula. In practice, however, unless the initial temperature is high, it will be found desirable to use the point represented by two retort temperatures, and find the other point by applying the formula to a value of CT of about 200°F. This choice of the second point is desirable because temperatures below 200°F. are not used in most General-Method calculations, and a graph extending down to a low initial temperature would be inconveniently large if it were to give accurate results at temperatures near the retort temperature.

When the retort temperature remains the same but the initial temperature is changed,

$$CT' = RT - \frac{RT - IT'}{RT - IT}(RT - CT).$$

This equation also represents a straight line and it is necessary to find only two points in order to draw it. If $CT = RT$ then $CT' = RT$ so that the point represented by the two (equal) retort temperatures may be chosen for one of the points. The point represented by $CT = IT$ and $CT' = IT'$ could be chosen for the other point, but here too, and for the same reason, it will be found more convenient to use a value of CT of about 200°F. in order to determine the other point.

If it is desired to change both the retort temperature and the initial temperature, the two formulas can be applied successively in either order.

Strictly speaking, these formulas should be used only for points on the heating curve, and points on the cooling curve should be transformed by different equations, but the error introduced by using the same formula for both heating and cooling is not of serious magnitude.

These formulas were originally derived on the assumption that heating is by conduction. Later, it was shown that the equations are also valid for "ideal" convection heating as described in the first paper of this series by Schultz and Olson (1938). In the case of vacuum-packed products, the use of these formulas becomes questionable since a mathematical theory of heat transfer in vacuum-packed products has not yet been developed.

SUMMARY

The construction and use of a special coördinate paper for thermal process calculations by the General Method has been described. With this paper, calculations may be made with less effort and the

chance of misplotting points is decreased. Formulas are also given for converting heat-penetration data obtained for one condition of initial temperature and retort temperature to corresponding data for different retort and initial temperatures. These formulas greatly increase the applicability of the General Method.

ACKNOWLEDGMENT

The authors are indebted to Dr. C. O. Ball and Dr. J. M. Jackson for their helpful criticisms and suggestions.

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THERMAL PROCESSING OF CANNED FOODS IN TIN CONTAINERS. IV. STUDIES OF THE MECHANISMS OF HEAT TRANSFER WITHIN THE CONTAINER

J. M. JACKSON AND F. C. W. OLSON
Research Department, American Can Company, Maywood, Illinois

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The first two papers of this series, by Schultz and Olson (1938) and Olson and Stevens (1939), described methods which facilitate calculation of thermal processes for certain types of non-acid canned foods. Specifically, these methods may be employed for any condition of can size, initial temperature, and retort temperature when the heat transfer within the can is known to be by conduction or convection, and the rate of heating is known for some particular set of conditions. The above methods of calculation are based upon simple heating curves, that is, time-temperature curves which, when plotted using a logarithmic scale in degrees below retort temperature, are asymptotic to a straight line.

Many foods which heat by conduction exhibit simple heating curves; for example, cream-style corn, pumpkin, potted meats, thick soups, and tomato juices of heavy consistency. Other foods, such as dilute broths, thin fruit juices, brine-packed peas, syrup-packed fruits, and evaporated milk, heat mainly by convection currents but also exhibit simple heating curves. However, a third group of foods, including the thicker soups, brine-packed whole-grain corn, certain tomato juices and a number of specialty or formulated products exhibit the so-called broken heating curve. Such curves when properly plotted are asymptotic to one straight line, then show a distinct change in heating rate and become asymptotic to a second straight line. The intersection of the two asymptotes is called the "break."

In most instances, the rate of heating after the break is slow and the form of the heating curve may show considerable variation from can to can and from batch to batch. Thus, selection of the proper data upon which to base process calculations for products exhibiting this type of heating curve is often a perplexing problem. In addition, methods used in the case of simple heating curves for converting the data from one condition of can size, initial temperature, and retort temperature to another are questionable when applied to broken heating curves.

Considerably more information is needed regarding the factors which might affect broken heating curves. Study of these factors

in the past has been largely limited by seasonal production of typical products exhibiting broken heating curves and by the can-to-can and batch-to-batch variations in the rate of heating described above. Consequently, some artificial system was sought which would be free from the above limitations and facilitate the study of the effects of controlled variables on the broken type of heating curve. After preliminary studies of aqueous suspensions of starch, methyl cellulose, and gum Karaya, suspensions of bentonite were selected as conforming more closely to the requirements of the desired synthetic system. It is the purpose of this report to describe the results of preliminary investigations on the temperature-distribution patterns in various bentonite suspensions, and also to describe the effect of some process variables on the broken heating curves exhibited by a certain type of bentonite suspension.

DEFINITIONS OF TERMS

To facilitate understanding of the data subsequently presented, a list of definitions of terms is presented here. For more detailed information, reference should be made to more comprehensive treatises, Ball (1923, 1927, and 1928) and Bigelow, Bohart, Richardson, and Ball (1920).

A typical broken heating curve is illustrated (Fig. 1). A curve

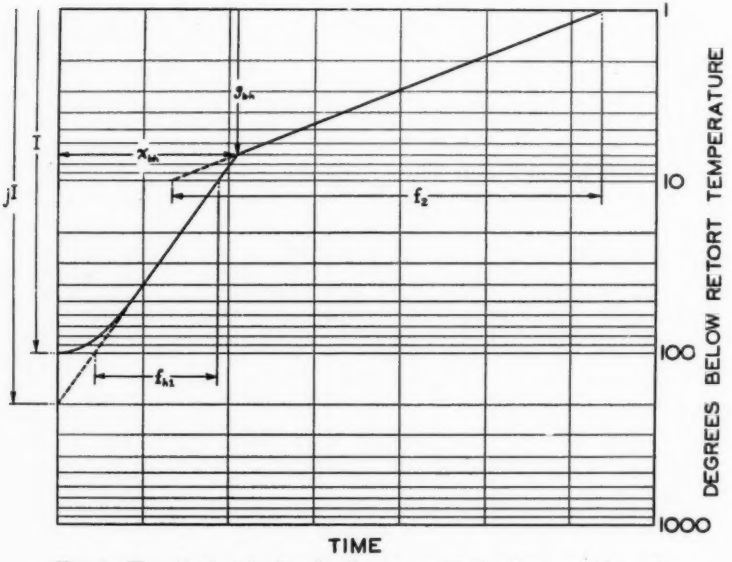


FIG. 1. Hypothetical broken heating curve illustrating symbols used.

is determined by the terms which have been defined by Ball (1923, 1927, and 1928) and are given below.

RT The retort temperature in °F.

IT The temperature at which the heating curve intersects the axis of ordinates. (Initial temperature.)

I The difference, RT — IT.

jI The difference between RT and the temperature at which the asymptote intersects the axis of ordinates.

j The ratio of jI/I. The value of j is a measure of the lag in heating rate at the start of the heating period.

f_{h1} The number of minutes required for the first asymptote to traverse one logarithmic cycle on the temperature scale.

x_{bh} The number of minutes from the start of the heating period to the break in the heating curve. The break is defined to be the point of intersection of the two asymptotes.

g_{bh} The difference between RT and the temperature at which the break in the heating curve occurs (this term is not needed to define the curve, but is useful).

f_2 The number of minutes required for the second asymptote to traverse one logarithmic cycle on the temperature scale.

The form and position of a simple heating curve are determined by RT, IT, j and f_h. The additional terms x_{bh} and f_2 are required to determine a broken heating curve.

EXPERIMENTAL PROCEDURE

Preparation of Bentonite Dispersions: Bentonite dispersions were prepared according to directions obtained from the supplier of the bentonite.[1] No attempt was made to purify the bentonite as received. Suspensions were prepared by adding the 300 mesh powder to distilled water in amounts corresponding to 1, 3.25, and 5 per cent, respectively, of the weight of water present. The settled clay was allowed to stand four hours and the mixtures were then agitated with a mechanical stirrer. A given suspension was used as many as nine times without appreciable change in heating characteristics. It was found necessary, however, to stabilize the suspensions by heating them in sealed cans to within one degree of the retort temperature, 121.1°C.(250°F.), before making the first heating test. Without this preliminary heat treatment, great changes in the heating rate between the first and second heat processes were observed.

Method: Heat penetration into the bentonite suspensions contained in No. 2 (307×409)[2] and No. 10 (603×700)[2] cans was

[1] The American Colloid Company.

[2] The designation of can size used in the industry is derived from the nominal can dimensions. The first digit represents inches, the next two the extra fraction expressed as sixteenths of an inch. The over-all diameter is cited first, followed by the over-all height.

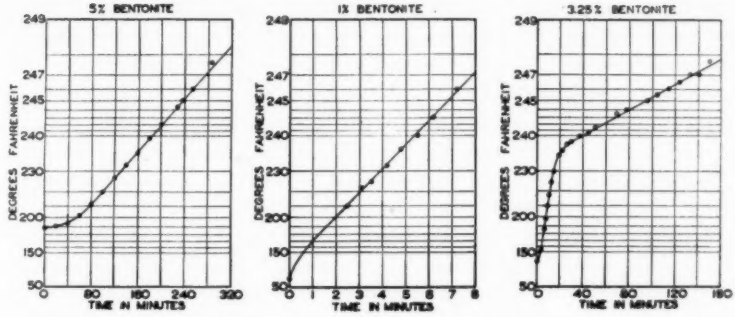
determined by the use of copper-constantan thermocouples inserted through the can walls by the use of ¼-inch bakelite rods and stuffing boxes. Several thermocouples were inserted in each can with the hot junctions located at various positions within the can as later described. The sealed cans containing the suspensions were processed in a vertical position in a small retort at holding temperatures of 104.4, 121.1, and 132.2°C.(220, 250, and 270°F.). About one minute was required to bring the retort to the desired holding temperature; however, instantaneous come-up time was assumed in plotting the curves. The required time corrections were made to compensate for the actual differences, Ball (1923, p. 45).

In most cases, the volumes of liquid filled into the cans were controlled to give net headspaces of ¼ and ⅛ inch at room temperature in the No. 10 and No. 2 cans, respectively. In the case of No. 2 cans, other headspace variables were also included. Uniform initial temperature—usually room temperature—throughout the can and contents was obtained at the start of each run although variable initial temperatures of 4.4 to 82.2°C.(40 to 180°F.) were included among the variables studied. Pressure effects were determined by the use of variable closing temperatures—room temperature to 85°C.(185°F.)—which gave gauge vacuums in the cans in the range of 0 to 16 inches, measured at room temperature. The possible effect of deformation of the tin-plate can was investigated by the use of rigid brass containers of the same internal dimensions as the No. 2 and No. 10 cans. Under the conditions of the experiment, it is believed that the effect of the slower rate of heat transfer through the thicker brass walls and ends would be negligible.

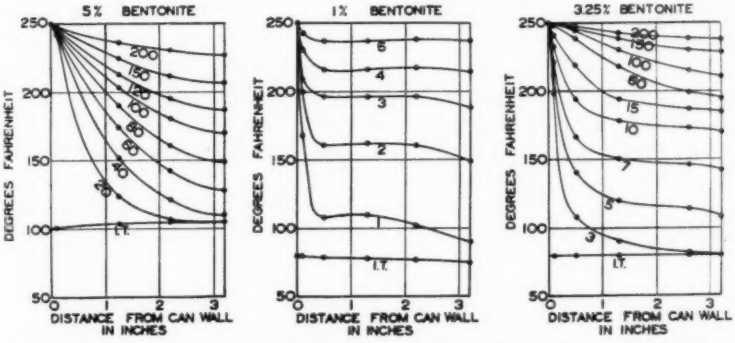
DISCUSSION OF RESULTS

Time-Temperature Curves and Temperature-Distribution Patterns: Typical time-temperature curves and temperature-distribution patterns observed within the can during the heating period are illustrated (Figs. 2 to 10); the results obtained with five per cent of bentonite, which gave simple heating curves with large f_h values (Figs. 2, 5, and 8); and the results obtained with one per cent of bentonite, which gave simple heating curves with small f_h values (Figs. 3, 6, and 9). Data obtained with tests on 3.25 per cent of bentonite, which gave broken heating curves, are presented (Figs. 4, 7, and 10). A typical heating and cooling curve for 3.25 per cent of bentonite is illustrated (Fig. 11). These data are from No. 10 cans only, but similar results were obtained on a smaller scale with No. 2 cans. The data warrant brief detailed discussion.

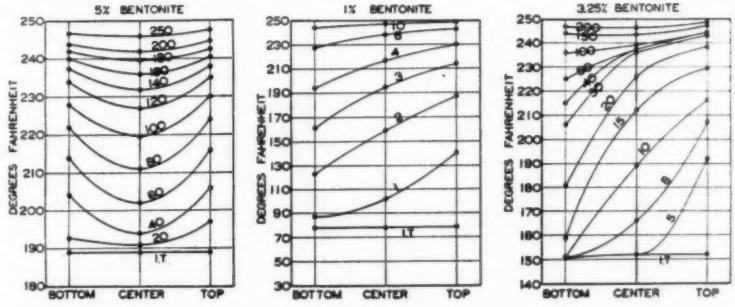
1. *Conduction Heating:* In the case of simple heating curves of



FIGS. 2, 3, and 4. Typical experimental time-temperature curves from thermocouples located 1.5 inches above the bottoms of No. 10 cans.



FIGS. 5, 6, and 7. Temperature-distribution patterns across central horizontal planes in No. 10 cans. (The curves represent temperature distribution at various designated minutes during the processes.)



FIGS. 8, 9, and 10. Temperature-distribution patterns along the vertical axes of No. 10 cans from 1.5 inches below the top to 1.5 inches above the bottom. (The curves represent temperature distributions at various designated minutes during the processes.)

large f_h values (Figs. 2, 5, and 8), the temperature distribution in the can indicates that heating is taking place by conduction. A graph plotting temperature against distance from the can wall (Fig. 5) shows a constant temperature portion near the center and rising temperature near the can wall during the lag period. After the lag period the temperature rises on a smooth curve from the center to the can wall, indicating that heat is conducted symmetrically from the outside of the can toward its center.

2. *Convection Heating:* Cans which exhibit simple heating curves of small f_h value (Figs. 3, 6, and 9) show a very different type of temperature-distribution pattern. The fact that the temperature rise takes place in the upper portion of the can first and that the lower portion is the last to reach retort temperature indicates that the heat is transferred mainly by convection currents in the liquid. Apparently the rising hot liquid forms a thin-walled tube adjacent to the can wall. As the hot liquid reaches the top of the can it spreads inward over the upper surface of the remaining liquid. The uniform temperatures shown across a horizontal plane indicate that the large cylinder of liquid within the rising tube is relatively quiescent. This cylinder must move down slowly as the liquid in contact with the bottom is heated and enters the rising tube, and the liquid flowing from the top of the tube replaces that at the top of the cylinder. Thus, the temperature rise in most of the can is due to the slow downward movement of hot liquid from the top of the can. Actually, the mechanism of heat transfer must be complicated by some eddy currents and some transfer by conduction.

As may be inferred (Fig. 9), the value of the lag factor j increases from the top to the bottom of the can. The numerical value of j was found to be of the order of .5 near the top of the can and approached unity near a point about an inch from the bottom. It is believed that under ideal conditions, a point could be found near the bottom where the value of j would exceed unity.

3. *Broken Curve Heating:* Before the break occurs the magnitude of f_h (Fig. 4) and the temperature-distribution pattern (Figs. 7 and 10) indicate convection heating.

After the break in the heating curve there is a lag period and then a new straight line heating curve of large f_2 value is established. The fact that temperature distribution in the can changes from that for convection heating and approaches that for conduction heating and also the fact that f_2 is of the same order of magnitude as f_h for conduction heating, indicate that the break in the heating curve is due to a sudden change from convection heating to conduction heating. This change takes place first in the upper part of the can

where the temperature differences and resultant forces owing to density differences are least. As the lower parts of the can contents become warmer, the change from convection to conduction takes place there also. The temperature at which the break occurs in the upper part of the can is somewhat higher than that for the lower part. The convection currents in the upper part of the can are probably prolonged by the relatively strong convectional forces which are present in the lower part of the can when the break is about to occur in the upper part.

The cooling curve associated with a broken heating curve is of special interest (Fig. 11). When cooling begins, the rate of tem-

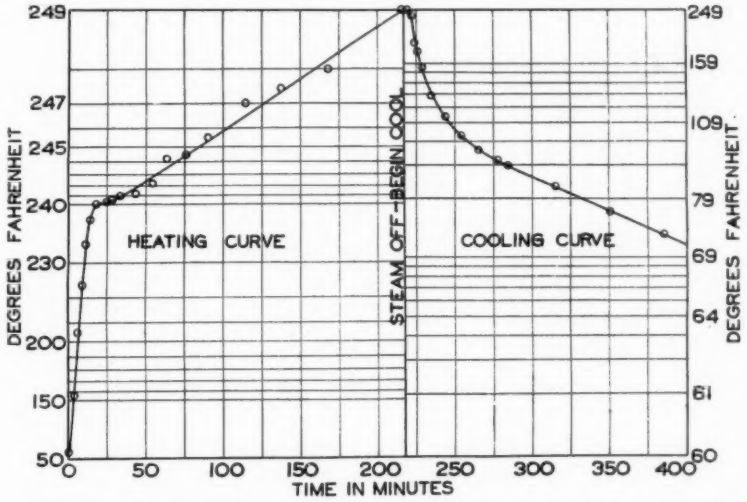


FIG. 11. Heating and cooling curves for 3.25-per cent bentonite suspension. Thermocouple 1.5 inches above bottom of No. 10 can.

perature drop is great; but as cooling continues, the rate decreases gradually to a constant value. There seems to be, however, no sharp break in the cooling curve corresponding to the break in the heating curve.

4. *Physical Properties of Bentonite Suspensions:* Bentonite suspensions are known to be thixotropic and rheopectic. These properties are well discussed by Freundlich (1937) and Hauser and Reed (1937).

Thixotropic suspensions form gels which become sols when they are subjected to physical shock and agitation. The sols will form gels again when they are allowed to stand for a time without severe

agitation. In rheopectic systems the sol-gel transformation is hastened by slight rhythmic agitation such as that caused by tapping a test tube containing the sol. The setting time decreases when the concentration of the suspension is increased. It has also been demonstrated that the setting time decreases with increase in temperature.

It is well known that a certain yield force must be exceeded to break a gel by the application of a shearing force. The effect of the reverse procedure of reducing the shearing force applied to a liquid has not been studied so thoroughly. Motion in a Newtonian liquid will not stop completely until the shearing force has been diminished to zero. However, since phenomena of anomalous viscosity have been observed in some liquids and since some sols are known to gel during very slight agitation, it is probable that when the shearing force applied to a thixotropic or rheopectic liquid is diminished slowly the liquid will set to a gel when the force applied has become less than some yield value, but still exceeds zero.

5. *Interpretation:* When a can containing a 3.25-per cent suspension of bentonite is first subjected to heat in the retort there are considerable temperature and density differences between the hot gel in contact with the container and the remaining cool gel. The resultant buoyant force is sufficient to exceed the yield point for the weak gel and start convection currents. As the cooler portions of the liquid rise in temperature the buoyant force becomes less and the convection currents diminish. In a Newtonian liquid one would expect that the currents would diminish steadily but would not cease until all of the liquid had reached retort temperature and the buoyant force had diminished to zero.

The rate at which the convection currents and heating rate diminish would also be dependent on the viscosity of the liquid, since the buoyant force is opposed by the viscosity. If the viscosity decreases with increasing temperature at a steady rate, the heating rate may be expected to change at a steady rate. This is compatible with a smooth time-temperature curve approximating a straight line when plotted on a semi-logarithmic coördinate paper.

The abruptness of change from convection heating to conduction heating may be explained by assuming that the buoyant force has decreased below the yield point for thixotropic or rheopectic gelation and that convection is stopped by sudden setting to a gel. This occurs first in the upper portions of the can, then the gel formation proceeds downward until convection has been stopped in all portions of the can.

It has been noted that when the retort temperature is raised, but other conditions are kept constant, the value of g_{bh} increases. The

liquid sets to a gel when the buoyant force is appreciably greater than that which will allow gelation when the retort temperature is lower. This indicates that the gelling tendency for the bentonite sol increases as the temperature of the sol is raised.

When cooling is started there are considerable temperature and density differences between the cooled gel in contact with the container and the remaining hot gel so that the convectional forces again become sufficient to exceed the yield point and start convection currents. The slow cooling rate which obtains toward the end of the cooling period indicates that gelation may have occurred again and that heat transfer is taking place by conduction. The fact that there is no abrupt change in cooling rate to indicate a sol-gel transition may be accounted for by one or more of the following factors which differ from the conditions during heating: The gradual decrease in convectional forces is accompanied by gradual increase in the viscosity of the sol; the temperature of the cooling medium in contact with the can changes gradually during the cooling period [Fig. 11 was plotted assuming the temperature of the cooling medium was constant at 15°C.(59°F.)]; and the gelling tendency is not so strong since the temperature is lower than at the time of gelation during the heating period.

Effects of Certain Variations in Heating the 3.25-Per Cent Bentonite Suspensions: As indicated in the preceding sections, a 3.25-per cent bentonite suspension exhibits the same broken type of heating curve as some food products and hence lends itself to a study of factors which might possibly influence this type of heat transfer.

In order to determine whether any of the variables normally encountered in canning practice affect the form of the broken time-temperature curves, a series of tests was made in which these variables were carefully controlled. The tests included the effects of flexibility of the container, headspace in the container, retort temperature, and initial temperature. The data were analyzed by statistical methods and the results are summarized (Tables 1 to 6).

TABLE 1

Effect of Flexibility of Container

Heating curve factor	Container		t value [1]	Conclusion
	Rigid	Tin plate		
j	1.16	0.99	1.33	Insignificant
f_{h1}	6.00	5.94	0.15	Insignificant
f_2	62.0	57.3	1.55	Insignificant
x_{bh}	7.8	7.0	1.44	Insignificant

[1] Least significant value of t is 2.179.

TABLE 2

Effect of Headspace

Heating curve factor	Net headspace		t value [1]	Conclusion
	$1\frac{1}{32}$ inch	None		
j	1.45	1.47	0.10	Insignificant
f_{h1}	4.87	5.97	2.09	Insignificant
f_2	45.5	45.0	1.62	Insignificant
x_{bh}	6.1	7.9	1.90	Insignificant

[1] Least significant value of t is 2.776.

TABLE 3

Effect of Retort Temperature

Heating curve factor	Retort temperature		t value [1]	Conclusion
	93.3°C.(200°F.)	132.2°C.(270°F.)		
j	1.22	1.07	1.43	Insignificant
f_{h1}	5.47	6.84	1.22	Insignificant
f_2	48.1	44.6	2.89	Significant
x_{bh}	8.33	4.58	14.8	Highly significant
g_{bh}	4.78	38.08	5.7	Highly significant

[1] Least significant value of t is 2.262; least highly significant value of t is 3.250.

TABLE 4

Effect of Initial Temperature

Heating curve factor	Initial temperature		t value [1]	Conclusion
	32.2°C.(90°F.)	4.4°C.(40°F.)		
j	1.22	1.30	0.63	Insignificant
f_{h1}	5.47	5.30	1.02	Insignificant
f_2	48.1	45.2	0.83	Insignificant
x_{bh}	8.3	8.9	0.76	Insignificant

[1] Least significant value of t is 2.776.

TABLE 5

Effect of Closing Temperature [1]

Heating curve factor	Can closed at		t value [2]	Conclusion
	21.1°C.(70°F.) (zero vacuum)	85°C.(185°F.) (16-inch vacuum)		
j	1.49	1.72	2.19	Insignificant
f_{h1}	4.9	4.0	2.04	Insignificant
f_2	50.5	54.2	0.84	Insignificant
x_{bh}	7.7	6.7	2.70	Significant
g_{bh}	9.3	9.3	0	Insignificant

[1] Initial pressure in the can. [2] Least significant value of t is 2.262; least highly significant value of t is 3.250.

TABLE 6

Effect of Can Size

Heating curve factor	Can size				t value [1]	Conclusion
	No. 2 experimental	No. 10 calculated for convection heating	No. 10 calculated for conduction heating	No. 10 experimental		
j	1.11	.....	.....	1.24	1.66	Insignificant
f_{hl}	5.81	.....	.....	10.5	7.64	Highly significant
f_{hl}	.....	10.2	.....	10.5	0.37	Insignificant
f_{hl}	.....	.....	18.1	10.5	6.08	Highly significant
f_2	55.9	.....	.....	163.0	16.80	Highly significant
f_2	.....	98.3	.....	163.0	7.80	Highly significant
f_2	.....	.....	174	163.0	0.83	Insignificant
x_{bh}	6.7	.....	.....	11.2	6.92	Highly significant
g_{bh}	15.7	.....	.....	18.9	0.88	Insignificant

[1] Least significant value of t is 2.052; least highly significant value of t is 2.771.

The data considered in these tables are from tests with 3.25 per cent of bentonite in the No. 2 can size. The heating curves are for thermocouples whose hot junctions were on the vertical axes of the cans, three-fourths of an inch above the bottoms. This location was found to be approximately the slowest heating point in the can when the broken heating curve was exhibited.

The data given in the tables are averaged values for the various heating curve factors taken from a number of tests. The tables also give a t value for each factor. These t values are calculated from the data by the method of Fisher (1934) and serve as criteria to determine whether the differences between the factors obtained under two different conditions are insignificant, significant, or highly significant, as shown in the last column of each table. The least significant and least highly significant values of t depend upon the number of observations made and have been determined by Fisher. For the purposes of this report it is not considered necessary to describe in detail the exact meanings of "least significant," "least highly significant," and "t value." Instead, merely the conclusions based upon their use are given in the tables.

From these tables we may conclude that reasonable variations in the rigidity of the container, in the headspace, or in the initial temperature will not cause any significant changes in the values of the heating-curve factors. Variations of the retort temperature, however, produce highly significant changes in the value of x_{bh} and g_{bh} but do not affect j or f_{hl}. The effect of the retort temperature on f_2 cannot be determined conclusively from the data. The closing temperature does not produce any highly significant changes in any of

the heating-curve factors. The effect of the closing temperature on x_{bh} is questionable.

As may be expected, variation in can size affects significantly the heating rates f_{hl} and f_2. To convert data from one can size to another, convection-heating factors should be used for f_{hl} and conduction-heating factors used for f_2. The factors j and g_{bh} do not seem to be affected by variation in can size, but x_{bh} does vary significantly with the can size.

SUMMARY

Studies were made on the heating rates and the mechanisms of heat transfer in tin cans using bentonite suspensions of various concentrations. The only variation in processing procedure that was found to have a significant effect on the form of the broken heating curve was variation in retort temperature from one process to another: The time at which the break occurs was found to vary inversely with retort temperature. The effects of variations in pressure within the container, flexibility of the container, headspace within the container, and initial temperature within the range studied were insignificant. The heating rates f_{hl} and f_2 and the time of break varied with the can size, but the temperature at which the break occurs and the lag factor j did not vary significantly with the can size.

The mechanism of heat transfer within cans has been found to fall into three classes: heat transfer mainly by conduction, heat transfer mainly by convection, and heat transfer mainly by convection during the early part of the process followed by heat transfer mainly by conduction during the remainder of the process. A theory has been developed that the change from convection to conduction in broken-curve heating is due to a sol-gel transition in the substance heated.

ACKNOWLEDGMENT

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RELATIONSHIP BETWEEN TIME OF FREEZING BEEF AFTER SLAUGHTER AND AMOUNT OF DRIP

J. M. RAMSBOTTOM AND C. H. KOONZ
Research Laboratories, Swift & Company, Chicago, Illinois

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Many investigations have been made on the drip associated with frozen-defrosted beef; however, there is very little available information on the relationship between drip and the time of freezing after slaughter.

Cook, Love, Vickery, and Young (1926) reported that in general the drip was less when the meat was held for a period not to exceed 10 days at 1°C.(33.8°F.) before freezing. Sair and Cook (1938) found that the amount of drip was affected by the time between slaughter and freezing and by the pH of the meat. These authors were principally concerned with the latter factor.

The purpose of the present investigation was to study the correlation between drip and the time of freezing beef after slaughter. Data were recorded on the pH of the beef, ice-crystal size, and amount of drip which was lost from the thawed product.

EXPERIMENTAL PROCEDURE

Three experiments were made in the present investigation. They are reported separately except for the following procedure, which was common to all experiments.

The beef was of select quality from the rib section of carcasses weighing approximately 600 pounds. The cuts were stored at 2.2°C. (36°F.) during the prefreezing period. Steaks one inch in thickness were cut from the longissimus dorsi muscle at specified intervals dating from the time of slaughter. The steaks were frozen at the temperatures stated in each experiment.

The steaks were thawed on wire screens suspended in 15-gallon tin containers in still air at 10°C.(50°F.), relative humidity of 90 per cent. Drip was considered to be the loss of weight occurring during a one and one-half day defrosting period. Since there would be some evaporation, the exact value for drip would be slightly less than the value obtained.

Determinations of pH values were made on all cuts before they were frozen and again after they were thawed by the use of a Beckman pH meter equipped with a glass electrode and calomel half-cell. Each determination was made on a representative 10-gram sample

of beef mixed with an equal amount of distilled water adjusted to 20°C.(68°F.). Since the pH values of the steaks before freezing were essentially the same as the values for the defrosted steaks, only the former values are recorded in the tables which follow.

EXPERIMENT 1

In Experiment 1, that part of the longissimus dorsi muscle located between the sixth and twelfth thoracic vertebrae was dissected from the right and left side of six carcasses four hours after they were slaughtered. Steaks were cut from the 12 muscles and frozen at —34.4°C.(—30°F.) at intervals of one-quarter, one, five, and ten days after slaughter. During the prefreezing period the muscles were stored in moisture-proof pliofilm bags at 2.2°C.(36°F.).

Data on the pH and drip on 11 of the 12 muscles (Table 1) show that the pH of the meat one-quarter day after slaughter was 5.9;

TABLE 1

Drip From Frozen-Defrosted Steaks—Experiment 1

Time of freezing after slaughter	Number of steaks	Drip from steaks frozen at —34.4°C. (—30°F.)	Standard deviation of drip	pH of beef before freezing	Standard deviation of pH
days		*pct.*			
¼	11	2.2	0.45	5.9	0.23
1	11	5.3	0.73	5.7	0.10
5	11	4.7	0.63	5.7	0.10
10	11	4.5	0.54	5.7	0.08

at one, five, and ten days after slaughter the pH was 5.6, 5.7, and 5.7, respectively. It will be observed (Table 1) that the drip was low for steaks frozen one-quarter day after slaughter, while it was comparatively high for steaks frozen at one, five, and ten days after slaughter.

One of the sides of beef used in this experiment was a dark cutter; that is, the beef failed to brighten in color after cutting. The muscle hemoglobin for the most part remained in the reduced state with consequent dark color rather than becoming rapidly oxygenated and developing a bright red color. Owing to the widely divergent values on the pH and drip of the steaks from this side of beef as compared with its companion side and the other three carcasses, a separate tabulation of the data is made (Table 2). It is interesting to note that the pH values of the dark cutting beef were always high, fluctuating between 6.2 and 6.5, and that the drip was practically negligible at one-quarter, one, five, and ten days.

TABLE 2

Drip From Frozen-Defrosted Dark-Cutting Steaks

Time of freezing after slaughter	Drip from steaks frozen at —34.4°C.(—30°F.)		pH of beef before freezing	
	Dark-cutting steaks, right side	Normal-cutting steaks, left side	Dark-cutting steaks, right side	Normal-cutting steaks, left side
days	*pct.*	*pct.*		
¼	0.8	2.1	6.2	5.8
1	0.7	4.5	6.2	5.7
5	0.8	4.2	6.4	5.9
10	0.7	4.3	6.5	5.8

EXPERIMENT 2

In Experiment 2, four commercial rib cuts were used. The ribs were stored at a temperature of 2.2°C.(36°F.) and a relative humidity of 80 to 85 per cent during the prefreezing period. At weekly intervals, during a five-week period, the ribs were faced to a depth of one inch, then boneless, fat-trimmed steaks were cut for the freezing tests.

A study of the data (Table 3) shows that there was a definite reduction in drip with each additional week that the beef was aged before freezing, while the pH remained uniformly low during the entire five-week period.

TABLE 3

Drip From Frozen-Defrosted Steaks—Experiment 2

Time of freezing after slaughter	Number of steaks	Drip from steaks frozen at —34.4°C. (—30°F.)	Standard deviation of drip	pH of beef before freezing	Standard deviation of pH
days		*pct.*			
7	4	5.7	1.37	5.5	0.05
14	4	4.5	1.34	5.6	0.07
21	4	3.1	0.55	5.6	0.00
28	4	2.1	0.37	5.5	0.07
35	4	1.8	0.27	5.6	0.05

EXPERIMENT 3

A third experiment was made for the purpose of obtaining more extensive data on the pH and drip of the meat as related to the time of freezing after slaughter. Rib cuts were selected from eight carcasses and stored at a temperature of 2.2°C.(36°F.). The relative humidity of the storage room was 80 to 85 per cent during the aging or prefreezing period. Boneless, fat-trimmed steaks were cut from the ribs at five days and 35 days after slaughter and frozen at tem-

peratures of —17.8°C.(0°F.) and —34.4°C.(—30°F.) according to the method already described.

Moisture and fat determinations, according to the Association of Official Agricultural Chemists (1935), were made on cross sections of muscle adjoining all test steaks, and corrections were made for differences in moisture and fat content between steaks from the same carcass cut at one and at five weeks after the animal was slaughtered. The average fat and moisture content of the samples taken at one week was 5.4 and 72, respectively, as compared with 6.1 per cent for fat and 71.1 per cent for moisture on samples taken from the ribs after they were aged for five weeks.

The percentage drip based on the frozen weight of the steaks and the pH of the beef before freezing are reported (Table 4). There was very little change in the pH of the beef during the 35-day period, the average values being the same and the variation was small as indicated by the standard deviations. Steaks frozen at —34.4°C. (—30°F.) were less subject to drip than steaks frozen at —17.8°C. (0°F.). The drip from the five-day aged steaks was approximately three times greater than the drip from the 35-day aged steaks.

TABLE 4

Drip From Frozen-Defrosted Steaks—Experiment 3

Time of freezing after slaughter	Number of steaks	Drip from steaks frozen at —34.4°C.(—30°F.) and —17.8°C.(0°F.)		Standard deviation of drip		pH of beef before freezing		Standard deviation of pH	
		—30°F.	0°F.	—30°F.	0°F.	—30°F.	0°F.	—30°F.	0°F.
days									
5	8	5.8	6.8	0.88	0.75	5.6	5.7	0.03	0.07
35	8	1.6	2.5	0.37	0.53	5.6	5.7	0.07	0.05

ICE-CRYSTAL SIZE AS RELATED TO TIME OF FREEZING AFTER SLAUGHTER AND DRIP

A study was made of the size, number, and distribution of ice crystals in representative frozen steaks as outlined in Experiments 1, 2, and 3 in accordance with the method described by Koonz and Ramsbottom (1939). In the present study, in addition to the paraffin sections, duplicate samples were placed in 75 per cent alcohol immediately after being removed from the dehydrating apparatus and subsequently treated according to the conventional celloidin method. Sectioning was accomplished more satisfactorily using the celloidin modification.

A microscopic study of the tissues showed that intrafiber freezing occurred (Fig. 1) when the steaks were frozen at —34.4°C.(—30°F.)

six hours after slaughter of the carcass. In steaks held for one day before freezing at —34.4°C.(—30°F.) extrafiber freezing was predominant, and this was also true for all subsequent intervals. The extra-fiber ice crystals in the steaks were rather small when frozen one day after slaughter (Fig. 2) but were progressively larger as the time between slaughter and freezing increased (Fig. 3).

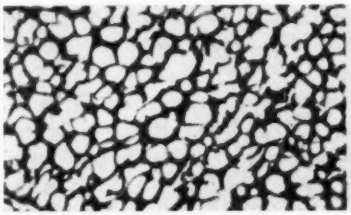

FIG. 1. Microscopic appearance of ice crystals in steaks frozen at —34.4°C. (—30°F.) one-quarter day after slaughter. X63.

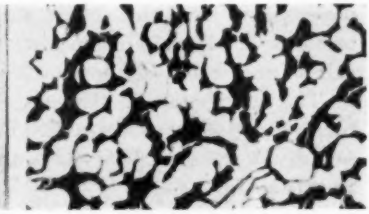

FIG. 2. Microscopic appearance of ice crystals in steaks frozen at —34.4°C. (—30°F.) one day after slaughter. X63.

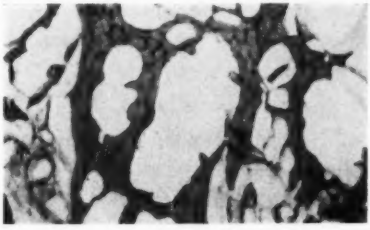

FIG. 3. Microscopic appearance of ice crystals in steaks frozen at —34.4°C. (—30°F.) 35 days after slaughter. X63.

DISCUSSION

It becomes apparent from a study of the data reported (Tables 1, 3, and 4) that drip decreases as the time between slaughter and freezing increases. An exception occurred (Table 1) with respect to the drip at one-quarter day. There was 2.2 per cent drip at one-quarter day and it increased to 5.3 per cent at one day. At one-quarter day the meat was still in rigor. The small amount of drip may be associated with the small intrafiber ice crystals. It is conceivable that the sarcolemma during rigor acts as a barrier preventing the loss of water from the fibers. This would be favorable to intrafiber freezing. The experimental steaks had a large cut surface in comparison with the volume of meat, and Ramsbottom and Koonz (1939) have shown that intrafiber freezing minimizes drip under these conditions.

After the first day the drip became progressively less, while the pH of the meat remained constant throughout its aging period. This must be interpreted to mean that the pH of the meat was not responsible for the decreased drip. It must be pointed out, however, that the hydrogen-ion concentration of the meat may be associated with the amount of drip as reported by Empey (1933) and by Sair and Cook (1938). Likewise, the data reported (Table 2) in the present paper show that the steaks with high pH values had negligible amounts of drip in comparison with the steaks wherein the pH values were relatively low.

The ice crystals in the meat became progressively larger as the time after slaughter increased (Figs. 1, 2, and 3), although the drip actually decreased. This shows that large ice crystals do not always accentuate drip.

The gradual decrease of drip as the time of freezing after slaughter increased must be attributed to changes which take place in aging meats. It is well known that progressive changes occur in the muscle proteins during the aging period. These changes apparently increase the capacity of the proteins to absorb water. Consequently, the "frozen out" water is more readily reabsorbed and drip is decreased.

SUMMARY

The present study was made on comparable steaks from select beef frozen at intervals during the first 35 days after slaughter. During the aging period the beef was held at 2.2°C.(36°F.).

The amount of drip from the steaks was found to decrease as the period of storage between slaughter and freezing increased.

The pH of the meat remained almost constant from the first day after slaughter to the 35th day after slaughter. Freezing and thawing did not significantly change the pH of the meat.

When the steaks were frozen at —17.8°C.(0°F.) and —34.4°C. (—30°F.), the ice crystals became progressively larger as the time between slaughter and freezing increased.

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EFFECT OF FERTILIZING TREATMENT ON VITAMIN A CONTENT OF SWEET POTATOES[1]

PEARL SWANSON, GLADYS STEVENSON,[2] E. S. HABER, AND P. MABEL NELSON
Iowa Agricultural Experiment Station, Ames, Iowa

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While soil fertility is known to affect size of harvest, flavor, and mineral content of foods, there is relatively little information concerning its influence upon vitamin content. In an early report, Honeywell and Dutcher (1930) stated that chlorotic spinach grown on soils deficient in manganese contained less vitamin A than normal spinach. Later, Tressler, Mack, and King (1936) observed that spinach grown in upland soil was richer in vitamin C than was spinach grown in muck soil. Ijdo (1936) found that the carotene and ascorbic acid contents of plants depended largely upon the nitrogen and potassium present in the soil. These studies were all made on a leafy plant. Since it was not known whether other types of vegetables were similarly influenced, the present investigation, dealing with the the effect of various fertilizing treatments on the vitamin A content of sweet potatoes, was initiated. The relation that soil fertility may hold to the nutritive value of a marketable crop of high-income value is an important problem to farmers in a section of Iowa where commercial fertilizers are commonly used.

EXPERIMENTAL PROCEDURE

The vitamin A content of sweet potatoes of the Prolific variety grown under five different conditions of soil fertility was determined by means of the bioassay. The Vegetable Crops subsection supervised the production of the potatoes. An area containing soil of a uniform composition (Buckner sand) in Muscatine County was used as the experimental plot. Buckner sand is deficient in nitrogen, phosphorus, potash, and organic matter. The area chosen was divided into five one-fiftieth acre plots. One of these plots was left in its original state and the other four were subjected to specific fertilizing treatments before the potatoes were planted. These respective plots received muriate of potash (800 lbs. per acre), manure (10 tons per acre), nitrate of soda (300 lbs. per acre), and superphosphate (500 lbs.

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[2] Now at the University of California at Los Angeles.

per acre). The outside rows of each plot served as guard rows, preventing contamination of any one plot by the fertilizers used on adjacent plots. After harvesting, the sweet potatoes were cured for two weeks at 26.7°C.(80°F.), then wrapped in paper, and stored at 11.7°C.(53°F.) in ventilated baskets.

The estimation of the quantity of vitamin A in each lot of potatoes was based on results obtained in three different assays. These 15 assays, involving a total of 146 rats, were conducted during the fall and winter months of 1932-33 and 1933-34. The technique used, based on the method originally outlined by Sherman and Munsell (1925), has been described previously by Swanson, Stevenson, and Nelson (1938). It included modifications suggested in the official method of the time—U. S. Pharmacopoeia X (1925)—and in the Interim Revision Announcement No. 2 of the Committee of Revision of the U. S. Pharmacopoeia (1934). Carotene supplied as a provisional standard material by the League of Nations was secured through the courtesy of Dr. E. M. Nelson of the U. S. Department of Agriculture in 1933. This substance was fed at different levels to groups of rats that were litter mates of the animals used in the potato assay.

The assay period was extended to eight weeks in order to secure data for the calculation of the Sherman-Munsell unit.

The sweet-potato supplement was fed to vitamin A-depleted rats six times per week, a double portion being given on Saturday. The potato was fed raw and the level of feeding was 33 mg. daily. One potato was fed over a period of three days, samples being taken successively from the ends and middle sections of the root. Wedge-shaped pieces were cut from circular slices taken each day from one of these sections and adjusted to a weight of $33 \pm .2$ mg. on the quantitative balance. After the first section was removed, the cut surface of the remaining portion was coated with paraffin to prevent evaporation. The cut potatoes were stored in an ice refrigerator.

The data were analyzed in terms of both the provisional international unit and the Sherman-Munsell unit. In the first analysis the curves of reference formulated for this purpose from the response of rats fed different quantities of carotene and already described by Stevenson and Swanson (1940) were used. The provisional unit thus obtained is equivalent to the present international unit because the vitamin activity of one gamma of the carotene distributed as a provisional standard is equal to that of .6 gamma of the beta carotene later available (McCollum, 1936). The international units were estimated from data obtained in eight-week and five-week assay periods.

The following equations, representing the curves of reference, were used in interpreting the results in terms of the provisional international standard:

(1) for an experimental period, 8 weeks long, $\bar{y} = (50.5x) + 2.99$;

(2) for an experimental period, 5 weeks long, $\bar{y} = (31.8x) + 9.71$.

In both equations $\bar{y}$ represents the average gain in weight made by a lot of rats fed a specific dose of A-containing food; and x, the estimated number of international units in the quantity of supplement fed.

RESULTS

The average gains in weight made by the lots of rats fed sweet potatoes grown under various conditions of soil fertility, with the standard deviations, are shown (Table 1) and these gains, interpreted in terms of international units and Sherman-Munsell units, in table 2. The differences in average gains in weight observed in the three assays based on any one type of sweet potato were found to be only those that might be expected from samples taken from a homogeneous population. The average concentration of vitamin A in each kind of potato was, therefore, calculated from the pooled results of the three assays (Table 3).

The concentration of vitamin A in the five kinds of potatoes ranged from 22 to 28 international units on the basis of the eight-week test and from 20 to 26 on the basis of the five-week test. The gains made by the animals fed potatoes grown under different soil conditions were not found to be statistically different in either the long or the short experiment (Table 1). Therefore, it was concluded that the values expressing unitage were not different either. Apparently, then, the fertilizers tested in the present investigation exerted no effect on the quantity of vitamin A present in the Prolific variety of sweet potatoes. This variety therefore contains on the average about 23.4 international units per gram. The number of Sherman-Munsell units per gram was 56.

The length of the experimental period did not affect the determination, since approximately the same results were obtained with the five-week assay as with the eight-week test. On the whole, however, results obtained in the three assays made with each kind of potato were less variable in the five-week test period than they were in the long period (Table 1).

The elimination, at the time supplementary feeding is initiated, of animals behaving erratically in the depletion period has been

TABLE 1

Average Gains in Weight Made by Lots of Depleted Rats Fed Sweet Potatoes Grown Under Different Soil Fertility Conditions

Analysis made	Fertilizer used	Assay I			Assay II			Assay III		
		Number of rats	Mean gain	Standard deviation	Number of rats	Mean gain	Standard deviation	Number of rats	Mean gain	Standard deviation
			gm.	*gm.*		*gm.*	*gm.*		*gm.*	*gm.*
Assay based on 8-week experimental period	Potash	13	39.9	14.1	8	44.4	14.2	10	40.3	9.8
	Manure	15	48.5	12.7	6	50.8	18.9	6	48.7	30.6
	Nitrate	17	37.4	13.1	4	45.5	6.8	7	46.0	12.7
	Phosphate	13	41.9	20.7	7	60.7	9.8	8	52.0	20.2
	No fertilizer	15	51.6	12.5	7	45.0	12.7	10	43.5	21.3
Assay based on 5-week experimental period	Potash	13	29.7	14.1	8	34.0	11.5	10	31.8	11.8
	Manure	15	36.3	10.5	6	35.8	15.6	6	41.5	23.7
	Nitrate	17	28.8	10.3	4	31.2	9.5	7	34.3	9.4
	Phosphate	13	32.7	17.1	7	40.4	8.0	8	40.1	16.2
	No fertilizer	15	39.4	10.7	7	36.0	15.0	11	32.8	15.5

TABLE 2

Units of Vitamin A (International and Sherman) in One Gram of Sweet Potatoes Grown Under Different Soil Fertility Conditions

Analysis made	Fertilizer used	Assay I				Assay II				Assay III			
		Total number rats used	Mean gain	Units of vitamin A in 1 gm. sweet potato		Total number rats used	Mean gain	Units of vitamin A in 1 gm. sweet potato		Total number rats used	Mean gain	Units of vitamin A in 1 gm. sweet potato	
				International	Sherman			International	Sherman			International	Sherman
			gm.				*gm.*				*gm.*		
Assay based on 8-week experimental period	Potash	13	39.9	21.9	49.8	8	44.4	24.6	55.5	10	40.3	22.2	50.4
	Manure	15	48.5	27.0	60.6	6	50.8	28.4	63.5	6	48.7	27.2	60.9
	Nitrate	17	37.4	20.4	46.5	4	45.5	25.2	56.8	7	46.0	25.5	57.5
	Phosphate	13	41.9	23.1	52.4	7	60.7	34.3	75.9	8	52.0	29.1	65.0
	No fertilizer	15	51.6	28.9	64.5	7	45.0	24.9	56.2	10	43.5	24.1	54.4
Assay based on 5-week experimental period	Potash	13	29.7	18.8	.....	8	34.0	22.9	.....	10	31.8	20.8	.....
	Manure	15	36.3	25.1	.....	6	35.8	24.6	.....	6	41.5	30.0	.....
	Nitrate	17	28.8	18.0	.....	4	31.2	20.2	.....	7	34.3	23.2	.....
	Phosphate	13	32.7	21.7	.....	7	40.4	29.0	.....	8	40.1	28.6	.....
	No fertilizer	15	39.4	28.0	.....	7	36.0	24.8	.....	11	32.8	21.8	.....

shown to increase significantly the uniformity of the assay and to decrease the expense, time, and labor involved—Swanson, Stevenson, and Nelson (1938). The present assays were used to test the procedure. In determining the average concentration of vitamin A in sweet potatoes by this plan, the data representing each assay

TABLE 3

Average Concentration of Vitamin A in One Gram of Sweet Potatoes

Fertilizer treatment	Eight-week test			Five-week test	
	Number of rats	Units		Number of rats	International units
		International	Sherman-Munsell		
Potash	31	22.7	51.5	31	20.5
Manure	27	27.4	61.3	27	26.1
Nitrate	28	22.4	50.7	28	19.6
Phosphate	28	27.6	61.9	28	25.5
No fertilizer	32	26.5	69.5	33	25.2
Average quantity of vitamin A in sweet potatoes	146	25.3	56.0	147	23.4

were pooled, since the gains made by the lots fed different kinds of sweet potatoes were not significant. Then the animals judged by regression to be erratic were eliminated, the total number of rats represented thereby being reduced from 146 to 84. The equation used was $\bar{y} = (31.8x) + 10.2$. The average concentration of vitamin A obtained from each assay compares very favorably with that based on all animals (Table 4). The usefulness of the plan for simplifying the test is thus indicated.

TABLE 4

Results of Bioassay When Selected Animals Only, Observed for a Five-Week Test Period, Are Used in Analysis of Data

Assay No.	Number of rats used	Mean gain of selected animals	Standard deviation	International units in 1 gm. of sweet potato calculated on basis of selected animals	International units in 1 gm. of sweet potato calculated on basis of complete data
		gm.	*gm.*		
I	50	36.9	13.5	25.2	22.3
II	15	32.1	8.7	20.7	24.6
III	19	32.2	14.8	20.8	24.2
Mean	84	34.9	12.8	23.4	23.4

DISCUSSION

The data presented herein indicate that the application of certain specific fertilizers did not influence the relative quantity of vitamin A present in a root vegetable grown in Buckner soil. The results

necessarily are not in disagreement with the work reported in the publications reviewed where spinach was the test material. Differences in vitamin A content, not demonstrable in storage organs, may occur in an actively functioning leafy system. Differences in vine growth, shape, and yield of roots were noted in the present experiment. The relative vitamin A content of the foliage system, however, is not always affected by a restriction of nutrients—Haber and Swanson (1935). Spinach, also, is a plant very sensitive to an unbalanced supply of nutrients, and whether or not other leafy plants respond similarly is a question. Finally, variations in vitamin A content attributed to fertilizing treatments may be demonstrated in sand cultures (Ijdo, 1936), where differences will not show up in field experiments. From the practical standpoint, no improvement in nutritive value can be affected by the addition of nitrogen, phosphorus, potash, or manure to a deficient soil.

The concentration of vitamin in sweet potatoes, as shown by the results of the present study (56 Sherman-Munsell units), is much higher than that observed by other workers. McLeod, Talbert, and Toole (1932) analyzed Nancy Hall sweet potatoes and found that they contained about 30 Sherman-Munsell units per gram. Hessler and Cole (1932) found only eight units per gram in the Nancy Hall variety, and Fraps and Treichler (1933a,b) observed only 20 to 40 units per gram when they analyzed sweet potatoes of the Porto Rico variety. The high unitage of vitamin A in the Prolific variety may be accounted for by the fact that its flesh is a deep yellow orange.

We were unable to find any report of the concentration of vitamin A of sweet potatoes in terms of the international standard.

CONCLUSIONS

1. The average concentration of vitamin A in sweet potatoes of the Prolific variety is 23 international units or 56 Sherman-Munsell units per gram.

2. The type of fertilizer used in the soil does not significantly affect the vitamin A content of the sweet potato produced.

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ERRATUM

Volume 5, No. 4, page 348. In Table 6, formula used, read "Cake flour.....300" instead of "Cake flour.....400."

SOME FACTORS IN THE SPOILAGE OF AN ACID CANNED FRUIT[1]

C. H. SPIEGELBERG
Pineapple Experiment Station, Honolulu, Hawaii

(Received for publication, December 18, 1939)

In the canning of acid fruits[2] considerations of color, flavor, and texture of the product suggest that processing should involve the minimum time and temperature of cooking that will produce sterility.[3] Taking advantage of the bacteriostatic action of relatively low pH, the accepted processes for acid products are mild in comparison with those required for nonacid vegetables. At times, however, as a result of deviations from accepted processes, variability of raw product or syrup, or increased contamination before processing, spoilage of acid canned fruits does occur.

Bigelow and Cameron (1932), Hallman and Stevens (1932), and Cameron (1938) indicate that spore-forming anaerobes, chiefly thermophiles, are the main causes of spoilage in nonacid products, but that in acid products the nonspore-forming bacteria are chiefly involved. Pederson (1929) concluded that in canned tomato products several species of lactic-acid bacteria were responsible although yeasts and spore-forming bacteria were encountered occasionally. Townsend (1929, 1938, 1939) found both nonspore-forming and spore-forming bacteria involved in spoilage of canned acid products. Berry (1933) found a flat-sour type of spoilage in canned tomato juice to be caused by a thermophilic, spore-forming, facultative anaerobe. Savage and Hunwicke (1923) reported *Escherichia coli* as the cause of spoilage in canned pineapple. The writer previously reported (1936) the occurrence of spoilage in canned pineapple caused by butyric anaerobes.

This paper, reporting studies completed several years ago, describes types of swells then observed in an acid fruit, *Ananas comosus* (L.) Merr., and considers the microörganisms responsible and the relationship of such factors as pH of raw product, buffering and pH of syrups, time and temperature of cooking, load of in-

[1] Published with the approval of the director as Technical Paper No. 132 of the Pineapple Experiment Station, University of Hawaii.

[2] "The upper limit of the acid range may be taken at pH 4.5. This is the point below which spore-forming bacteria cease to be factors in spoilage and it becomes unnecessary to use a process sufficiently severe to destroy them."—Bigelow and Cameron (1932).

[3] Sterility as used in this paper refers to commercial sterility—Esty and Stevenson (1925); and Ball (1938)—rather than to bacteriological sterility.

oculum, temperature of can storage, and occurrence of leaks, as they contribute to the occurrence of swells. Of particular interest is the demonstrated activity of a spore-forming anaerobe, *Clostridium pasteurianum*, in producing bursting swells in an acid product.

TYPES OF SWELLS

Six types of bacterial swells, encountered in canned pineapple, are dealt with here, omitting from consideration the rare yeasty swells seen only in grossly undercooked or leaky cans. The bacterial swells, caused by six distinct organisms or groups of closely allied organisms may be distinguished by joint consideration of amount of gas (degree of swelling) and the odor, consistency, turbidity, pH, and acidity of contents. Both the organisms and the swells they cause, established by numerous inoculations of processed cans,[4] are designated here by letters or numbers.

Types 7, A, B, and C, here grouped as "arrested swells," are characterized by little gas [from "flippers" to "soft swells" as defined by Esty and Stevenson (1925)], sometimes only enough to break the vacuum. None, however, is strictly a "flat sour." Of these four, Type 7 produces the most gas, the most acid (.20 to .41 normal), the lowest pH (3.3 to 3.5), and the greatest turbidity. Type A, with distinctly less gas, tends also to lower acidity (.16 to .24 normal), slightly higher pH (3.4 to 3.6), and lower turbidity. Type B resembles A in amount of gas but is distinguished by distinctly lower acidity (.08 to .12 normal) despite only slightly higher pH (3.5 to 3.7), and by still less turbidity. Finally, Type C, which resembles A and B in gas production and resembles B in turbidity, is distinguished by somewhat the least acid (.06 to .11 normal) and distinctly the highest pH (3.7 to 4.4). Although ranges given indicate some overlapping, few individual cans fall near these limits where classification becomes uncertain until checked by microscopic or cultural tests.

Buckling and bursting swells are of two readily distinguished types. In Type 240, swelling develops within 24 to 48 hours after processing; the gas is chiefly carbon dioxide resulting in foaming; the odor is sour; and the syrup is frequently slimy or sometimes like crystallized honey. Butyric swells develop more tardily, sometimes after a week or two; the gas is predominantly hydrogen; and the odor is cheesy. Both types are characterized by relatively low acidity and high pH.

[4] Transfers from these cans to beef-extract broth before inoculation showed many of them to contain viable organisms, mostly aerobic spore-formers of the *Bacillus subtilis* type, but numerous attempts to reproduce spoilage with these organisms gave negative results.

SPOILAGE ORGANISMS

Three of the six organisms have been identified. The butyric organism is *Clostridium pasteurianum* Winogradsky, including both rough and smooth forms, Spiegelberg (1940). Type 240 is *Leuconostoc mesenteroides* (Cienkowski) Van Tiegham, and Type 7 is *Lactobacillus plantarum* (Orla-Jensen) Bergey *et al.* These latter two determinations have been checked by Dr. C. S. Pederson who has described strains from tomato products, Pederson (1929) and Pederson and Breed (1929).

The A, B, and C type bacteria have not been identified and will be described only briefly. All are nonspore-forming, gram-positive rods. A and C are motile by means of peritrichous flagella and are catalase positive; B is nonmotile and catalase negative. None liquefies gelatin, produces indol, or reduces nitrates to nitrites. All produce acid in litmus milk, without coagulation. On potato the C type produces a moderate moist growth, tan to light brown in color, while the others produce little or no growth. A wide variety of carbohydrates was fermented with production of acid but no gas in beef-extract broth. All three types utilized dextrose, levulose, galactose, mannose, maltose, sucrose, lactose, mannitol, and sorbitol. In addition to these, the A and C types were positive on glycerol; the B and C types on raffinose, dextrin, starch, salicin, and amygdalin; while the C type alone utilized xylose, arabinose, rhamnose, and inulin.

Despite failure of A, B, and C, and also Type 7[5] to produce visible gas from carbohydrates in beef-extract broth, all produce some gas in pineapple juice. The butyric organisms and the 240 type produce abundant gas within 24 to 48 hours, while 7, A, B, and C produce a few bubbles to a few millimeters of gas under vaseline seal. In general, the rate and amount of gas produced by the several organisms in pineapple juice corresponds to the types of swells produced by them in the canned product.

Thermal Death Time: In preliminary tests, mixtures of the several organisms were heated in beef-extract broth in sealed tubes at 70°C.(158°F.) for 10 minutes. Only the butyric organisms survived. Similar results were obtained with naturally contaminated pineapple juice containing both spore-forming and nonspore-forming organisms and adjusted to pH 4.2, 5, and 6 before heating.

Heat resistance of Types 7, 240, A, B, and C was obtained by heating one cubic centimeter of a culture in sealed microtubes. Cul-

[5] *Lactobacillus plantarum* (Type 7) produces only small quantities of carbon dioxide from hexose sugars, Pederson (1938), but does produce visible swelling in canned tomato products, Pederson and Breed (1929, p. 7).

tures were grown in beef-extract broth plus two per cent dextrose, transferred to sterile broth, adjusted to pH 5.8, and transferred to triplicate microtubes. Positive and negative points were plotted on semi-logarithmic paper, Bigelow (1921). Typical results for a single strain of each of the culture types (Fig. 1) show that the most resistant organism, Type C, was killed by one minute at 70.5°C. (158.9°F.) and 10 minutes at 64.5°C.(148.1°F.). The least resistant organism, Type B, was killed by one minute at 60.5°C.(140.9°F.) and by 10 minutes at 52.5°C.(126.5°F.). The results obtained with Types 7 *(Lactobacillus plantarum)* and 240 *(Leuconostoc mesenteroides)* agree in general with those reported by Pederson (1929).

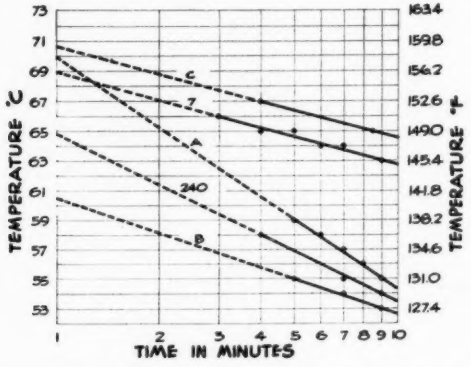

FIG. 1. Thermal death-time curves for a single strain of each of the non-spore-forming spoilage bacteria. The solid line connects the majority of points at which minimum time and temperature combinations produced sterility and the broken line is an extension of this curve.

The curve for the A-type culture seems out of line, but the same slope of curve was obtained for several other strains of this organism.

No attempt was made to define a thermal death-time curve for the butyric cultures, since these organisms resist time and temperature combinations which, if maintained in the can, would cause unfavorable changes in the canned product. Instead the effect of numbers of spores and the effect of pH of the medium used for heating and for culturing after heating were tested.

Spore suspensions obtained by washing agar-slant cultures with beef-extract broth, were diluted with sterile broth in various proportions to give the desired number of spores per cubic centimeter, based on a count of the first dilution only. Cultures were heated in pH 5 broth in sealed tubes, five tubes for each combination of pH, temperature, and dilution, and then transferred to pH 6.5 and 5

broth (Table 1). Following some of the heat treatments, growth occurred in broth at pH 6.5 but not pH 5, which indicates that in the canned product pH may be an important factor in producing commercial sterility when the organisms are not killed by the process. The R-type culture apparently is less heat resistant than the S type, although slightly smaller numbers of spores were present at the different dilutions.

The heat resistance of these butyric cultures is such that it is impractical to attain sterility in the canned product by killing the organisms, especially when large numbers are present. The non-spore-forming spoilage organisms, on the other hand, have a comparatively low heat resistance and, under the conditions of these tests, were killed by time and temperature combinations which are ordinarily surpassed in commercial practice.

Incubation Temperature: The occurrence of the several types of swells, sometimes weeks after processing, in warehouse stacks where the temperature ranged from approximately 27 to 29°C.(80 to 85°F.), indicated that thermophilic organisms were not involved. This was borne out by experimental packs which were cooled to approximately 38 to 43°C.(100 to 110°F.) and then incubated at room temperature.

Commercially-sterile cans inoculated with unheated cultures of the various organisms and incubated at temperatures ranging from 26 (room) to 55°C.(78.8 to 131°F.) showed that, in general, the higher temperatures increased the rate of swelling but did not materially affect the final percentage of swells.

To measure growth in relation to temperature, the organisms were inoculated into beef-extract broth and incubated at 10, 21, 26, 30, 33, 38, 43, and 55°C.(50, 69.8, 78.8, 86, 91.4, 100.4, 109.4, and 131°F.). The butyric cultures were incubated in this broth under vaseline seal and on anaerobic agar slants. The other cultures were incubated aerobically. All types grew within the range 26 to 43°C., although the most favorable range for all types was 30 to 38°C. Types 7, 240, and A showed some growth at 10°C., the B type and the butyric cultures at 21°C., while the C type failed to grow below 26°C. This latter type showed optimum growth at 43°C. and was the only one that grew at 55°C.

The results clearly indicate that temperature of incubation, within usual storage temperatures, is not an important factor limiting development of the types of spoilage investigated.

Relation to pH: The effect of pH on growth of active cultures was determined by inoculating commercially-sterile cans selected to provide a pH range from 3.7 to 4.8. Without further processing,

TABLE 1

Effect of Numbers of Spores on Heat Resistance of Butyric Organisms, Types S and R.

Heat treatment	No. 31 (S)										No. 57 (R)									
	Test medium										Test medium									
	pH 6.5 broth (2 tubes each)					pH 5.0 broth (3 tubes each)					pH 6.5 broth (2 tubes each)					pH 5.0 broth (3 tubes each)				
	Dilutions [1]					Dilutions					Dilutions					Dilutions				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
80°C.(176°F.)																				
10 min.	+[2]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+2
20 min.	+	+	+	+	+	+	+	+	+1	+	+	+	+	+	+	+	+	+	+	0
85°C.(185°F.)																				
10 min.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+2	+1
20 min.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+2	+1	0
90°C.(194°F.)																				
10 min.	+	+	+	+	+	+	+	+	+	+1	+	+	+	+	+	+	+1	+1	0	0
20 min.	+	+	+	+	+	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0
95°C.(203°F.)																				
10 min.	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[1] Dilutions	No. 31	No. 57
1 approximately	56,000,000 spores per c.c.	48,000,000 spores per c.c.
2 approximately	5,600,000 spores per c.c.	4,800,000 spores per c.c.
3 approximately	1,400,000 spores per c.c.	1,200,000 spores per c.c.
4 approximately	140,000 spores per c.c.	120,000 spores per c.c.
5 approximately	14,000 spores per c.c.	12,000 spores per c.c.

[2] + = all tubes positive; 0 = all tubes negative; +1 = number of tubes positive when some were negative.

these cans were incubated at room temperature. All those inoculated with culture Types 7, 240, and A swelled regardless of pH, but, among those inoculated with Types B and C and butyric cultures, all below pH 4.1 and most below pH 4.4 remained sound. These results indicate that pH of the can contents may be important in limiting growth of certain of the spoilage bacteria, especially the butyric organisms, since only spores would survive an ordinary cooking process and these would require a higher pH for germination than would the vegetative cells for growth (Table 1).

FACTORS RELATED TO SPOILAGE

Tests in commercial canneries indicated that the processes employed had a good margin of safety when fruit of average pH was used. When such fruit was packed with commercial juice syrups and given a standard process, the cans remained sterile, and only when the time or temperature of cooking was greatly reduced from the standard did spoilage develop. These results indicated that some factor other than temperature was involved.

The presence of swells caused by spore-forming butyric anaerobes and published data by Bigelow and Cathcart (1921) suggested that pH might be a factor of considerable importance. The data on relationship of pH to growth of the unheated cultures is indicative.

Experimental Methods: Data on the relationship of pH, temperature, and other factors to sterilization were obtained both in commercial canneries and in an experimental cannery which employed exhaust box, double seamer, rotary cooker, and water cooler. Heating was in steam at 100°C.(212°F.) and cans (size 2½) were cooled in running water to approximately 37.8 to 46.1°C. (100 to 115°F.). Temperature of can contents, from cooker and cooler, were measured by glass-stemmed thermometers inserted into the slices. In some tests, cans were packed with fruits showing wide variations of pH and acidity; but in most of the tests, fruits were selected for uniformity of appearance and composition; and a range of pH from about 4 to 5 was obtained by adding varying amounts of sodium citrate to the syrup. While results of experimental packs are based on average pH and temperature readings, it is felt that the conclusions drawn from them are valid within rather narrow limits.

Leaky Containers: Although in examining swells from commercial packs some visibly defective containers have been found, these have been few with yeasty spoilage predominating, in contrast with the larger problem of bacterial swells. Because of the possibilities suggested by Hallman and Stevens (1932, p. 659), however, an experimental study of the effects of cooling in contaminated water was

undertaken. Selected fruit was packed with buffered syrups, processed to bring fruit temperature to 93.3 to 96.5°C.(200 to 206°F.), then cooled in tanks of water with or without added cultures. The water was initially about 27.2°C.(81°F.), and cans remained immersed until cooled to 37.8 to 43.3°C.(100 to 110°F.).

TABLE 2

Cooling Cans in Contaminated Water, and Its Effect on Percentage[1] and Types of Swells Which Developed

Test No.	Average temperature of fruit after heating	Approximate pH of cans	Inoculation of cooling water	Swells	
				Arrested	Butyric
	°F.			pct.	pct.
1	200	4.9	0	0	47
			A, B, C	4	52
			Butyric	3	56
2	200	4.8	0	5	32
			A, B, C	11	42
			Butyric	2	32
3	200	4.6	0	0	4
			A, B, C	8	21
			Butyric	8	25
4	206	4.6	0	0	0
			A	0	0
			B	0	0
			C	0	0
			Butyric	0	0

[1] 24 to 74 cans in a series.

The data show that among cans which attained a temperature of 96.5°C.(206°F.) no swells occurred even after cooling in heavily inoculated water, but that after only 93.3°C.(200°F.) swells developed among cans cooled in both clean and contaminated water (Table 2). There are indications, however, the significance of which may be doubtful, that cooling in contaminated water may have had some influence upon swelling. Among Tests 1, 2, and 3 (Table 2), both butyric and arrested swells tended to be more numerous after cooling in water inoculated with either class of bacteria. This parallels the evidence (Table 7) that an addition of cultures of arrested swell organisms before processing increases the prevalence of butyric swells, but its meaning is obscure.

These experiments demonstrate that entry of bacteria through leaks was not the chief factor controlling occurrence of swells even

here, and limited sampling of both air and cooling water in commercial canneries failed to detect the presence of the spoilage organisms. In contrast, such bacteria are abundant in cans when sealed. Consequently attention was directed to factors influencing the effectiveness of cooking processes and determining the ability of organisms that survive the process to begin growth and cause swells.

pH of Raw and Canned Pineapple: The average pH of fancy sliced pineapple taken from commercial packs was 3.8, but the range

TABLE 3

Relation of pH and Temperature to Sterilization of Fruit Selected With Indicator Test Paper and Packed With High and Low pH Commercial Syrups

Test No.	pH of syrup	Process [1]		Swells
		Time	Temperature	
		min.	*°F.*	*pct.*
1	5.2	8.9	240 [2]	15
	4.3	8.9	240	0
2	5.2	15	210 [3]	92
	5.2	20	210	25
	5.2	25	210	50
	5.2	30	210	33
	4.3	15	210 [3]	83
	4.3	20	210	42
	4.3	25	210	33
	4.3	30	210	0
3	5.2	11.0	220	8
	5.2	11.0	226	8
	5.2	11.0	232	6
	4.3	11.0	220	0
	4.3	11.0	226	0
	4.3	11.0	232 [2]	0

[1] All cans vacuum sealed. [2] Standard process. [3] Boiling-water bath, no agitation of cans.

was from 3.6 to 4.4. In cans taken from packs in which spoilage had occurred the pH extended even higher, with occasional cans up to pH 4.7. Since all these cans had been packed with a pineapple-juice syrup of uniform pH, it appeared that the pH variations between cans were caused by variations in the fruit.

Use of an indicator paper test [6] in the cannery permitted the detection of raw fruits of high pH. Some 700 fruits, selected from

[6] Among several dyes tested, p-Sulfo-o-Methoxybenzene-Azo-Dimethyl-a-Naphthalamine was selected as the most suitable using .1 gram of the dry indicator in 100 c.c. of 95 per cent alcohol.

approximately 20,000 tested with the paper, included many of pH 4.5 to 4.8 and a few up to pH 5.2, compared with about 3.8, the pH of average fruits. These fruits also averaged low in titrable acidity although there was no direct relationship between this and pH. At that time field-run fruits averaged .7 to .9 per cent acid (calculated as citric) but, with few exceptions, selected fruits fell below .5 per cent acid, the lowest recorded being .13 per cent.

Effect of Syrups on pH and Sterilization: To determine the influence of syrup upon the pH of can contents after processing, selected fruits of pH 4 and above were sliced and packed, one fruit per can, with commercial pineapple-juice syrups of pH 5.2 and 4.3. Comparing percentages of raw fruits and of processed cans that were in the critical range of pH 4.5 and above, the high pH syrup

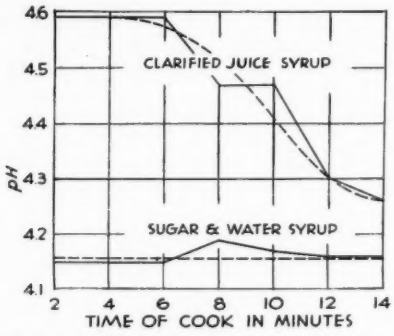

FIG. 2. Change of pH in juice and water syrups drained from a uniform pack of fruit after heating as indicated. Solid lines represent actual curves in each case, while the broken lines represent smooth curves.

increased the number of cans in this pH range by 55 per cent, whereas the low pH syrup *decreased* the number of cans in this same range by 31 per cent.

That these differences are significant in relation to sterilization is shown in the results of three typical tests (Table 3).

In Tests 1 and 3, which employed standard processes, the differences are clear-cut since swells occurred only when the high pH syrup was used. In Test 2, where the cans were cooked in a boiling-water bath without agitation, the effect of the pH of the syrup was not noticeable until after heating 30 minutes when no swells developed in the cans packed with low pH syrup.

The almost complete absence of spoilage in commercial packs employing unbuffered water syrup and its occurrence in cans packed with pineapple-juice syrup, which is highly buffered, led to a com-

parison of these two syrups in regard to their influence on pH changes during and following the process. Tests in a commercial cannery showed that the sugar and water syrup drained from the fruit had a lower pH than the juice syrup drained from the same type of fruit throughout the process. To determine more closely the magnitude and duration of these differences during the cooking process, cans packed with fruit, showing minimum variations of pH and titrable acidity, and water or juice syrup were heated in a pan of boiling water for 2, 4, 6, 8, 10, 12, and 14 minutes. Comparative pH readings of the drained syrup were made on one can of each series immediately after heating.

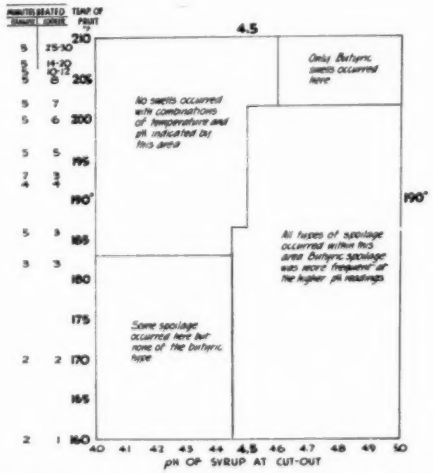
4.5
Only Butyric swells occurred here
No swells occurred with combinations of temperature and pH indicated by this area
All types of spoilage occurred within this area Butyric spoilage was more frequent at the higher pH readings
Some spoilage occurred here but none of the butyric type
190°
4.0 4.1 4.2 4.3 4.4 4.5 4.6 4.7 4.8 4.9 5.0
pH OF SYRUP AT CUT-OUT

FIG. 3. Effect of temperature and pH on occurrence and type of spoilage in experimental packs.

The buffered juice syrup was higher in pH than the unbuffered water syrup throughout the cooking process (Fig. 2). The difference was .45 pH during the first six minutes of heating, but after 14 minutes the difference had been reduced to .1 pH.

The influence of these differences on sterilization time was shown in two tests in which the cans were cooked in boiling water without agitation. In the first test, swells resulted after five to 20 minutes' heating with both syrups; however, the types of swells which occurred indicated that the water syrup was more effective than the juice syrup. In the second test shorter time intervals were used and the effectiveness of the water syrup is clearly indicated by the fact that 21 minutes were required to attain sterility, while 25 minutes were required with the juice syrup.

The effect of these two syrups with fruit of varying initial pH and with a process in which cans are rotated has not been determined, but it is felt that the results offer a plausible explanation for absence of spoilage in cans packed with water syrup.

Temperature: The effect of temperature and pH on the development of spoilage, determined from numerous tests, is shown in a simplified form (Fig. 3). Inoculated and uninoculated cans are included. When the pH was below 4.5, the cannery standard of 87.8°C. (190°F.) in the fruit from the cooker was adequate to insure sterility. With pH 4.5 and above, a temperature of 93.3°C. (200°F.) was required to eliminate the arrested swells, while butyric swells persisted with even much higher temperatures.

TABLE 4

Temperature in Relation to Amount and Types of Swells—pH Range 4.9 to 5

Inoculation	Temperature in fruit from cooker	Percentage [1] of swells which developed			
		Arrested	240	Butyric	Total
	°F.				
None	160	11	89	0	100
	170	16	53	16	85
	185	47	16	32	95
	192	68	0	16	84
A, B, and C types mixed	160	5	95	0	100
	170	47	32	11	90
	185	74	0	16	90
	192	58	0	26	84
Butyric	160	0	16	84	100
	170	0	21	79	100
	185	0	0	100	100
	192	0	0	100	100

[1] 19-can series.

When the pH of the can contents was approximately 4, temperatures much below 190°F. gave sterility. In one test, with an average temperature of 71.1°C. (160°F.) in the fruit following processing, two swells of the No. 240 type and five of the No. 7 type developed out of 38 cans processed, or five and 13 per cent, respectively. Cans which showed average temperatures of 76.7 and 82.2°C. (170 and 180°F.) remained sound. Occasionally, however, spoilage may be expected with a temperature of 180°F. in the fruit when pH is 4, as shown by another test where two arrested swells, four per cent, developed out of 50 cans processed. In general these tests confirm the statement of Bigelow and Cameron (1932) that sterilization of

acid products "may be accomplished by relatively short processing in boiling water sufficient to give a minimum temperature of 82°C. (180°F.) in the coldest part of the contents."

With similar processes and a high pH condition the results were much different as shown in the upper part of Table 4, which gives results with uninoculated cans. The 240-type swells *(Leuconostoc mesenteroides)* developed only with substandard processes, temperature of contents below 190°F., while arrested and butyric swells developed with both standard and substandard processes. The percentage of both arrested and butyric swells, but not of total swells,

TABLE 5

Effect of pH and Inoculation on Percentage[1] of Arrested and Butyric Swells

Test No.	Average temperature of fruit from cooker	Inoculation	Average pH of cans											
			4.0		4.2		4.4-4.5		4.6		4.8		4.9	
			A[2]	B	A	B	A	B	A	B	A	B	A	B
	°F.													
1	192	0	0	0	0	0	0	8	0	0	4	63	0	100
		A, B, C	0	0	0	0	6	13	0	42	8	92	0	100
2	194	0	0	0	0	0	0	0	0	0	0	33	..	..
		A, B, C	0	0	0	0	0	1	0	10	0	22	..	..
3	200	0	0	0	0	0	0	0	..	..	2	4	..	..
		A, B, C	0	0	0	0	0	0	..	..	2	15	..	..
		Butyric	0	0	0	0	2	0	..	..	0	100	..	..

[1] 12 to 72 cans in each lot. [2] A = arrested swells; B = butyric swells.

increased with increased processes, owing to the fact that the 240-type swells occur only when the lower processes are used and develop much faster than the other types. With increased processing the 240 type is eliminated, then the slower developing types are arrested, and butyric types appear.

A further analysis of the effect of temperature and pH on spoilage is shown (Table 5), uninoculated series, which summarizes the results of three tests given different processes, all of which gave average temperatures in the fruit above 190°F. Below pH 4.4 to 4.5, no swells developed and the 240 type is completely absent even at high pH. The percentage of arrested swells shows no clear-cut relation to pH but the increase in butyric swells with increase of pH is readily apparent.

Effect of Inoculation: Several tests showed that when large numbers of butyric organisms were added to high pH cans (pH 4.8-5) before processing, the invariable result was a large percentage

of butyric swells. This is illustrated by comparing (Table 4) the cans receiving no inoculum and those receiving butyric spores; Test 3 (Table 5) presents a similar comparison. In one test, heating for 30 minutes following a five-minute exhaust failed to produce sterility, although four minutes' heating after this exhaust gave an average temperature of 190°F. in the fruit. It is evident that when a combination of high pH and heavy inoculum occurs it is not feasible to control the butyric spoilage by increasing the time of cooking.

That the influence of inoculation of cans is proportional to numbers of spores added is shown (Table 6). Chance inoculum was apparently heavy, for with no spores added, 50 per cent swells followed eight minutes' cooking of these high pH cans. Additions of 3,300 spores per can resulted in no increase but when 66,000 or more spores

TABLE 6

Effect of Varying Numbers of Butyric Spores and Time of Process on Development of Butyric Swells in High pH Cans

Series	Number of spores added, per can	Cooking time			
		8 min.	12 min.	15 min.	20 min.
A	26,400,000	100 [1]	96	67	8
B	1,320,000	100	79	46	0
C	66,000	100	63	4	0
D	3,300	38	8	0	0
E	0	50	0	0	0

[1] Percentage figures based on 24-can series.

were added, 100 per cent swells followed eight minutes of cooking. With progressively longer periods of cooking, the percentage of swells fell off, leaving a clearly defined tendency for heavier inoculation to give more swells. Also swelling was delayed by long cooking and accelerated by increasing inoculation. First swells appeared four days after processing, and last swells after 13 days.

With swells caused by nonspore-forming bacteria, the effect of inoculation is less striking (Tables 4 and 5). Some increase in percentage of arrested swells was obtained by inoculating with A, B, and C types, but results were not consistent.

While inoculation with A, B, and C types did not greatly affect the percentage of arrested swells which developed, this treatment did increase the prevalence of butyric swells when inoculated and uninoculated series were compared (Table 7). An appreciable increase in butyric swells in inoculated over uninoculated cans occurred in all but one experiment, the pH 4.9 series to which a great excess of sodium citrate was added, resulting in 100 per cent swells in both inoculated and uninoculated cans.

While unexplained, this phenomenon seems significant as it occurred on four different days with two times of heating and at 4 pH values.

DISCUSSION

The amount and kinds of spoilage found during this study indicate, in the light of experimental results, that pH has played a rôle of major importance. With butyric spoilage, pH 4.5 seems to be a critical point below which spoilage does not occur and above which

TABLE 7

Increase in Percentage[1] of Butyric Swells by Inoculating Cans With A, B, and C Types of Spoilage Organisms as Compared With Uninoculated Cans

Date of test	Average temperature of fruit after heating	Approximate pH of cans	Inoculation	Swells
	°F.			pct.
7- 6-34	200	4.8	0	4
			A, B, C	17
7-11-34	200	4.8	0	4
			A, B, C	13
7-18-34	192	4.8	0	42
			A, B, C	92
		4.9[2]	0	100
			A, B, C	100
7-25-34	192	4.5	0	13
			A, B, C	21
		4.6	0	0
			A, B, C	42
		4.8	0	83
			A, B, C	92

[1] 12 to 24 cans in a series. [2] 80 grams of sodium citrate were added per 1,000 grams of syrup in this series while only 40 grams per 1,000 were required to reach pH 4.8.

it may or may not occur depending on numbers of organisms present and time and temperature of cooking. The small percentage of butyric swells and their delayed appearance in commercial packs indicate that these swells resulted from a high pH condition, with relatively small numbers of the butyric organisms present per can. That occasional high pH fruits were often responsible for the high pH condition is indicated.

While pH below 4.5 in itself is sufficient to control the butyric swells it is not as effective with some of the other types of spoilage since the organisms causing them are able to grow even in the lowest pH cans and must be killed by the process. Here again pH is im-

portant since these types are more easily killed at or below approximately pH 4.4 than above this point (Fig. 3). At these high pH values the cannery standard of 87.8°C.(190°F.) in the fruit leaving the cooker is not sufficient to prevent the occurrence of the arrested swell types.

The 240-type swells, however, occurred only when the temperature failed to reach 190°F. during processing. This was true in even the highest pH cans, so that the presence of this type of swell definitely indicates gross understerilization or leaky containers. The occurrence of a few cans of this type of swell probably indicates leaky containers but when numerous cans of this type appear, understerilization is probably the cause.

The various spoilage organisms are present in sufficient numbers at most times to cause spoilage when other conditions are favorable, but their elimination before entering the can does not seem feasible or necessary. However, the greater prevalence of butyric swells when large numbers of these organisms were added to the cans and also the effect of the arrested swell organisms in increasing the prevalence of the butyric swells, points to the necessity of keeping the numbers of spoilage organisms at a minimum. The butyric organisms do not readily build up large heat-resistant populations in the cannery. The nonspore-forming spoilage organisms, however, readily find suitable conditions for rapid development, the 7 and 240 types especially, since they are least restricted by the average acidity of fresh pineapples.

SUMMARY

Factors involved in the spoilage of an acid canned fruit caused by both spore-forming *(Clostridium pasteurianum)* and nonspore-forming *(Lactobacillus plantarum, Leuconostoc mesenteroides)* and three unnamed types of bacteria are discussed.

Commercial processes were found effective when fruit of average pH was packed with commercial pineapple-juice syrups. Swells developed only when the time or temperature was greatly reduced from the standard. However, when the pH of the can contents was increased by either adjusting the pH of the syrup or by selecting high pH fruits and packing them with unadjusted commercial syrups, pH was found a factor of considerable importance in sterilization. A low pH juice syrup was more effective in producing sterility than a high pH juice syrup. Unbuffered water syrup was more effective than naturally buffered juice syrup.

With pH of 4.4 to 4.5 and below, 87.8°C.(190°F.) in the fruit following the cooker was adequate to insure sterility, but with pH

4.5 and above a temperature of 93.3°C.(200°F.) was required to eliminate the nonspore-forming types of swells, while butyric swells persisted with even much higher temperatures.

The effect of inoculation on prevalence of the various types of swells is considered.

On the basis of experimental results, control reduces itself to maintaining the final pH of cans at or below 4.4 and processing to give a temperature of 190°F. or above in the fruit from the cooker.

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CHEMICAL AND PHYSICAL CHARACTERISTICS OF THE PETROLEUM ETHER SOLUBLE MATERIAL OF FRESH AND CANNED FLORIDA VALENCIA ORANGE JUICE[1]

ARTHUR J. NOLTE AND HARRY W. VON LOESECKE
U. S. Citrus Products Station, Bureau of Agricultural Chemistry and Engineering, U. S. Department of Agriculture, Winter Haven, Florida

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Orange juice preserved by canning deteriorates in flavor during storage. The reason for this deterioration is not known with certainty, but it is generally agreed by authorities that such off-flavors are due in part to oxidation. The newer technique of deaëration and high-short pasteurization as compared with older methods of processing has greatly improved the quality of canned orange juice, but, nevertheless, after several months' storage at ordinary temperatures off-flavors will develop.

Nelson, Mottern, and Eddy (1933), believing that the nitrogenous constituents of the juice, which amount to about one per cent of the total solids, might throw some light on the cause of the flavor deterioration undertook a study of the nitrogen bodies of fresh and aged, canned orange juice. No changes were detected in the nature of the constituents present in the deteriorated juice from those in fresh juice.

These authors point out that it is highly probable the development of off-flavors is due to some constituent or group of constituents present in small amounts. The fatty matter of orange juice, alleged to be present in amounts averaging about .2 per cent according to Chatfield and McLaughlin (1931), might fall within this category. It was thought that off-flavor development may be caused by rancidity changes of this fat. The literature contains no references relative to the investigation of the fatty material of orange juice. Matlack (1929) has, however, made a study of the non-volatile constituents of the rind of (California) oranges. It is a well-known fact among whisky distillers that an extremely small amount of rancid fat in whisky will impart a disagreeable odor to the product.

In order to verify the theory that rancidity may cause off-flavors in canned orange juice, preliminary tests were conducted in samples of fresh juice and canned juice seven months old, which had a distinct off-flavor. The samples were extracted with ethyl ether, the

[1] Food Research Division Contribution No. 480.

ether solution was washed with water, and the extracts, after removal of solvent, were examined organoleptically. The fresh-juice extracts had a bland taste and fragrant odor, while extracts from the canned juice had an unpleasant, rancid taste and a stale, tallowy odor. Täufel and Thaler's (1932) ketone test when applied to the distillate from canned juice gave positive results, while a negative test was obtained in a distillate from fresh juice. Ketone rancidity can be detected by taste when methyl nonyl ketone or methyl heptyl ketone are present to the extent of only four parts per million, Schmalfuss, Werner, and Gehrke (1933).

EXPERIMENTAL PROCEDURE

Since preliminary investigations indicated that rancidity actually occurred in aged, canned orange juice, a more detailed investigation of the problem seemed warranted.

Florida Valencia oranges were used and the juice extracted by reaming them over slowly revolving burrs. This method of extraction is comparable to that now being used commercially. The juice used for investigation of the fresh fatty material had a Brix of 9.6°, a total acidity of 1.10 per cent as anhydrous citric acid, and a pH of 3.82. The juice used for canning had a Brix of 8.8 to 11°, total acidity of .96 to 1.29 per cent, and a pH of 3.69 to 3.53.

The juice was canned by deaërating, using a Pulley (1936) deaërator, screening through a 20-mesh screen, and pasteurizing at 90.5°C. (195°F.) for five seconds in a tin-coil pasteurizer, quickly cooling to 85°C.(185°F.), and filling into plain cans at this temperature—Von Loesecke, Mottern, and Pulley (1934). The sealed cans were rapidly cooled and stored for 16 months at a temperature of 26.7 to 40.5°C.(80 to 105°F.).

Extraction of Fatty Material: One hundred forty-two liters (approximately 37.5 gallons) of the freshly reamed juice was immediately centrifuged four times by means of a Sharples Supercentrifuge, and the yellow, pasty material, which deposited in the centrifuge bowl, was collected. Even though the juice was centrifuged four times it still possessed a light-yellow color which would indicate that some extractive matter was not removed. To remove all of the extractive matter would have required more time than the slight additional yield would justify.

The thick, pasty material obtained was extracted several times with acetone [2] and then about 40 times with petroleum ether. The acetone extracts were evaporated to dryness and dissolved in petro-

[2] Acetone was first used to partially remove the water and hence make easier subsequent extractions with petroleum ether.

leum ether. The petroleum ether extracts were combined, partially evaporated, filtered, and washed seven times with distilled water. The precipitate obtained consisted of about .4 gram of a waxy substance.

The washed petroleum ether extract was evaporated *in vacuo* and heated *in vacuo* at 80°C.(176°F.) until all solvent had been removed. Thirty-five grams (amounting to a yield of .024 per cent based on the original juice) of a reddish-brown solid of buttery consistency were obtained.

One hundred twenty-two liters (approximately 32 gallons) of the 16 months' old canned juice were centrifuged and extracted in the same manner as described above for fresh juice. Fifty grams (amounting to a yield of .034 per cent based on the weight of the canned juice) of a reddish-brown, thick liquid was obtained.

TABLE 1

Characteristics of Petroleum Ether Extract of Fresh and Aged, Canned Florida Valencia Orange Juice

	Fresh juice	Aged, canned juice
Index of refraction $N_D20°C$	1.5078	1.4825
Acid value	23.5	77.4
Iodine No. (Hanus)	87.9	73.7
Saponification value	142.8	183.8
Insoluble acids (Hehner)	76.3	58.0
Peroxide value (M)	0.006	.30
Kreis test	Negative	Positive
Higher ketone test (Täufel)	Negative	Positive
Issoglio oxidizability number	4.5	13.8
Aldehyde test (Schibstead)	1.7	Nil
Apparent acetyl value	14.9	125.1

Examination of Petroleum Ether Soluble Material: The extract obtained from fresh juice had a sweet, fragrant odor and a bland, oily taste; that obtained from the aged, canned juice possessed a stale odor and a rancid, bitter taste. The extract from fresh juice was a buttery solid at room temperatures; while that from aged, canned juice was a thick liquid at room temperatures (Table 1).

Saponification of Fat and Separation of Saturated and Unsaturated Acids: To a weighed portion of the extract from fresh juice was added an excess of .5N alcoholic potassium hydroxide and the mixture was refluxed for four hours. The alcohol was removed by distillation, water was added, and the soaps extracted seven times with petroleum ether; this extract was reserved for examination of unsaponifiable constituents.

The aqueous soap solution was acidified with dilute hydrochloric acid and the mixture extracted seven times with petroleum ether. The acid layer was observed to contain a reddish-brown solid and was reserved for future examination of resins and phytosterolines. The petroleum ether was washed, the wash waters being added to the acid layer above, and the petroleum ether removed by distillation. The residue obtained was dried at 100°C.(212°F.) under a blanket of carbon dioxide. It contained 36.9 per cent solids composed chiefly of free fatty acids. The residue was taken up in alcohol, a slight excess of alcoholic potash was added, and the separation of the saturated and unsaturated fatty acids was carried out by the lead salt-ether method. The total free fatty acids obtained had an iodine number of 111.2 and amounted to 31.5 per cent of the total solids, the difference between this figure and 36.9 per cent obtained above being considered as resinous material.

Because of the profound change that had taken place in the characteristics of the extract from the aged, canned juice, separation of unsaturated and saturated acids could not be satisfactorily accomplished by the lead salt-ether method. The oxidized extract contained appreciable quantities of hydroxy acids (shown by the high apparent acetyl value, Table 1) which are readily converted to lactones when subjected to the treatment called for in the lead salt-ether method. Lactones, being neutral compounds, show an apparent decrease in neutralization values. Since lactones are readily soluble in ether and alcohol, they affect the separation of the fatty acids from the resins causing an apparent increase in the fatty-acid fraction and a decrease in the resin fraction. The values obtained are, however, significant in that the differences between the saponification and neutralization values are due to the hydroxy acids becoming dehydrated and forming inner anhydrides, characteristic in the oxidation of fatty acids, Lewkowitsch (1921a). In spite of this, the lead salt-ether method was used for the separation of the unsaturated and saturated fatty acids in aged, canned juice. The total free fatty acids amounted to 44.9 per cent, a higher value than obtained for the fresh juice because of the presence of resins and lactones. Characteristics of the free fatty acids from fresh juice and aged, canned juice are given (Table 2).

The constants obtained for the free fatty acids in aged, canned juice show that separation was not clear-cut and confirms the hypothesis regarding the apparent presence of lactones.

Further attempts were made to obtain a clean separation of the acids by converting the petroleum ether soluble material from the aged, canned juice into ethyl esters. The esters were diluted with

water, heated to boiling, cooled, and extracted with petroleum ether. The petroleum ether was washed with water and then treated with .2N potassium hydroxide and a small amount of alcohol. The petroleum ether solution of the esters was floating on top of a watery layer of resin soaps. The esters were washed with water, excess solvent was removed by distillation, and the residue was dried at 100°C. in a blanket of carbon dioxide. The dried esters were then extracted with methyl alcohol and filtered. The methyl alcohol extract was acidified with hydrochloric acid and treated with petroleum ether. The petroleum ether extract was washed free of acids, the solvent was removed by distillation, and the residue was dried at 100°C. under carbon dioxide. The constants obtained for the fatty acids separated by this method were essentially the same as those

TABLE 2

Characteristics of Free Fatty Acids of Fresh and Aged, Canned Florida Valencia Orange Juice

	Fresh juice	Aged, canned juice
Neutralization number	173.4	152.5
	pct.	*pct.*
Saturated acids (corrected)	6.90	13.43
Unsaturated acids (corrected)	78.40	57.28

obtained for the fatty acids separated by the conventional lead salt-ether method. It is therefore concluded that the method described is no more efficient than the lead salt-ether method.

Composition of Unsaturated Acids: The iodine number of the unsaturated acids of fresh juice was 118.5. When aliquot portions of an ether solution of the unsaturated acids of fresh juice were brominated in the cold, no hexabromide precipitate was observed. This fact proved the absence of not more than faint traces of linolenic acid. Since, therefore, the unsaturated acids consisted essentially of only oleic and linoleic acid, the percentage of these acids was calculated according to Lewkowitsch (1921b); 30 per cent of linoleic acid and 70 per cent of oleic acid were obtained.

As previously stated, a clean separation of the unsaturated and saturated acids could not be obtained in the case of aged, canned juice. It was therefore impossible to identify the unsaturated acids.

Composition of Saturated Acids: The iodine number of the saturated acids from fresh juice was 6.33. Insufficient material was available to attempt a quantitative separation of the saturated acids of the fresh juice. Identification was, however, attempted, by recrystallizing the mixed acids from alcohol-acetone solutions. An acid was

obtained melting between 82 and 85°C.(179.6 and 185°F.). The acid was converted into its potassium salt which was found to be insoluble in cold water. Since the potassium salt of cerotic acid is insoluble in cold water, this fact, together with the melting point of the acid, indicates that this factor is composed chiefly of cerotic acid.

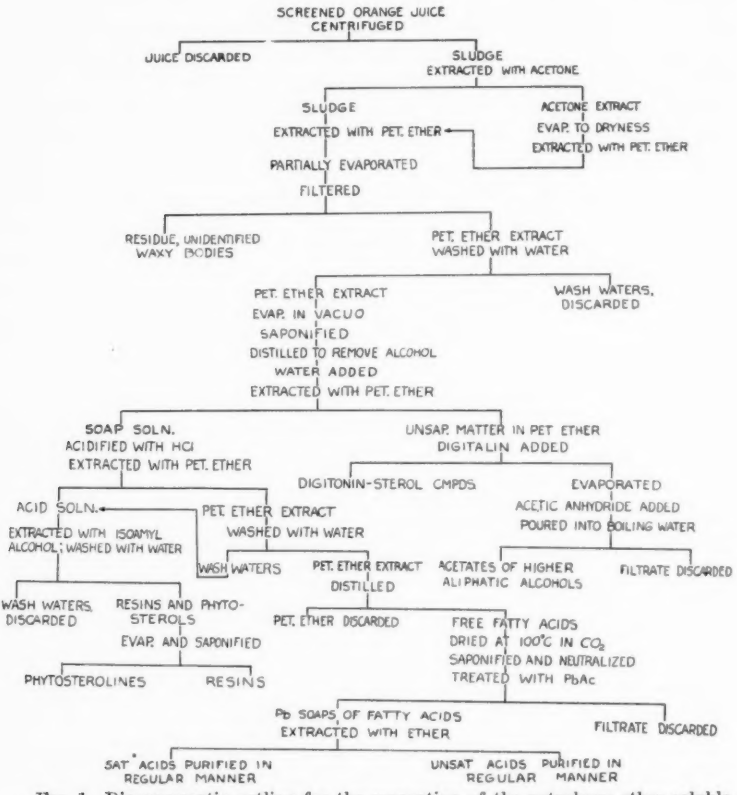

FIG. 1. Diagrammatic outline for the separation of the petroleum ether soluble constituents of orange juice.

Further crystallizations of the mother liquor obtained after removal of cerotic acid yielded a crystalline acid melting between 65 and 69°C.(149 and 156.2°F.). Stearic acid melts between 69 and 71°C.;(156.2 and 159.8°F.); palmitic acid, between 62.6 and 63°C. (144.6 and 145.4°F.). The crystals obtained were apparently a mixture of these two acids, consisting chiefly of stearic acid.

The mother liquor from these crystallizations was evaporated to dryness, the crystals obtained melting between 56 and 59°C.(132.8

and 138.2°F.). This was considered to be a mixture of stearic and palmitic acid, chiefly the latter.

Although the saturated acids obtained from the aged, canned juice were impure because of reasons previously stated, attempts were made to identify the acids by repeated recrystallization from ethyl alcohol. Two fractions were obtained—one melting at 73 to 75°C.(163.4 to 167°F.) and the other at 53 to 55°C.(127.4 to 131°F.). Their molecular weights were calculated from the neutralization value, but they did not correspond to the molecular weights of any known fatty acid. It is probable these acids were so grossly contaminated with lactones and resins, their clean separation would be virtually impossible.

Extraction of Resins: This fraction has been called resins because of the solubility of the material in various solvents, and its saponification, iodine, and acid values, all of which are within the range of substances classified as resins.

TABLE 3

Constants of Resins Separated From Fresh and Aged, Canned Florida Valencia Orange Juice

	Fresh juice	Aged, canned juice
Per cent of resin [1]	53.8	36.4 [2]
Acid number	33.9	93.5
Saponification number	72.1	126.6

[1] Based on total petroleum ether extract. [2] Apparent percentage.

The liquid containing reddish-brown material after the extraction of the fatty acids from fresh juice with petroleum ether was extracted with isoamyl alcohol. The alcohol was washed several times with water, the solvent removed by distillation, and the residue dried at 100°C.(212°F.). A dark-red solid of buttery consistency was obtained, equivalent to 53.8 per cent of the total solids. When fused with potassium hydroxide, negative results were obtained for phenols. No nitrogen was found present.

The characteristics of the resins obtained from the aged, canned juice were quite different from those found in fresh juice (Table 3).

Inasmuch as the resins obtained from aged, canned juice contain lactones resulting from the chemical treatment afforded the hydroxy acids during separation, as explained above under the discussion of the separation of the free fatty acids, they are not true values. The percentage of resins in the aged, canned juice appears to be less than it actually is because a portion of the resins was extracted and included in the free fatty-acid fraction owing to the solubility of the lactones in petroleum ether. Thus, the percentage of free fatty

acids in the petroleum ether extract from fresh juice was 31.5 per cent, while from aged, canned juice it was 44.9 per cent.

Isolation of Phytosterolines: A portion of the resinous material from fresh juice was shaken with an excess of .5N sodium hydroxide and the mixture allowed to stand until the solids had disintegrated. The mixture was filtered, the precipitate washed with water until free of alkali, dried, and weighed. The phytosterolines thus obtained amounted to .3 per cent, based on the weight of the petroleum ether extract. The presence of phytosterolines was confirmed by refluxing with amyl alcohol and dilute hydrochloric acid, neutralizing with sodium carbonate, and boiling with Fehling's solution. The Liebermann-Burchard reaction for sterols was also positive.

The same procedure as above was carried out on the resinous material from aged, canned juice, .4 per cent being obtained. Considering that separation is not strictly quantitative, it can be said there is no difference in the phytosteroline content of fresh and aged, canned juice.

Insufficient material prevented positive identification of these phytosterolines.

Determination of Carotinoid Pigments: Carotinoid pigments were determined according to the colormetric method of Sprague (1928), using napthol yellow and orange G as a standard. The petroleum ether soluble extract of fresh juice contained .11 per cent carotinoid pigments.

Isolation of Phytosterols: The unsaponifiable material obtained from the soap solution by extraction with petroleum ether amounted to 5.04 per cent of the total extract in the case of fresh juice, and to 1.77 per cent of the total extract in the case of aged, canned juice.

A one-per cent alcoholic solution of digitonin was added to the unsaponifiable matter, the solution shaken, warmed, and allowed to stand overnight. Ethyl ether was added to the alcoholic solution, the precipitate filtered off, washed first with alcohol and then with ether, dried, and weighed.

The phytosterol equivalent of the digitonide obtained was approximately .2 per cent of the total extract for both fresh and aged, canned juice.

The presence of sterols was confirmed by the Liebermann-Burchard reaction.

The filtrate from the digitonite was evaporated to remove the solvent, acetic anhydride added, and the mixture refluxed. The material dissolved completely in the hot acetic anhydride, indicating the presence of aliphatic alcohols. The acetylated mass was poured into boiling water to hydrolyze the excess acetic anhydride, allowed

to cool, and the precipitate was filtered, washed free of acid, and dried. The acetate of the higher alcohols thus obtained amounted to .48 per cent of the total extract in the case of fresh juice. In the case of the extract from aged, canned juice, results were so erratic they were considered unreliable.

The quantity of the acetates was too small to attempt identification.

DISCUSSION OF RESULTS

The petroleum ether extract obtained from fresh orange juice had a bland taste and aromatic odor, while that from aged, canned juice had a stale, tallowy odor and a rancid taste with bitter aftertaste.

The fatty material of orange juice undergoes profound changes when the juice is canned and stored at elevated temperatures. That such changes are primarily brought about by oxidation is shown by the fact that the refractive index, iodine number, and insoluble acids decreased; while there was an increase in acidity and saponification value. There was an increase from .006 to .30 in the peroxide value (M). Whereas the fatty material from fresh juice showed no Kreis test, that obtained from aged, canned juice showed a positive reaction.

It will be noted, too, that the extract from aged, canned juice indicated higher ketones. This is significant in that minute amounts of the higher ketones can be detected by their unpleasant taste. The presence of these higher ketones was not only established in the extract of the fatty material but also in a distillate from aged, canned juice.

An increase in the Issoglio oxidizability number of the extract from aged, canned juice indicates that oxidation created volatile decomposition products.

The aldehyde test was negative in the case of the rancid extract of the aged, canned juice. This may be explained by the fact that when a fat is oxidized in the presence of moisture, acids are the final products. In fact, extreme oxidation of fats is characterized by the absence of aldehydes.

The high acetyl value of the fatty material from aged, canned orange juice indicates that the oxidized fats and resins have been partly changed to hydroxy acids.

From the above facts it is quite apparent that the fatty material in orange juice undergoes oxidative changes when such juice is canned according to present methods and subjected to storage. Since it has been shown that rancidity results in a change of flavor of the fat and the creation of oxidative products, it is quite reasonable

to suppose this phenomenon would result in off-flavors in the canned juice. Whether or not oxidation of the fatty material is entirely responsible for deterioration in flavor of canned orange juice cannot be stated in the light of the evidence presented here. It is, however, reasonable to assume that such oxidation of the fatty material assumes an important rôle in the development of off-flavors.

The data do not indicate how these oxidative changes are brought about, but they may result through oxygen left in the headspace of the container [3] or through enzyme activity. During the process of extracting the juice it is possible, too, that the fat in the juice absorbs oxygen to form loosely bound oxygen compounds, which Holm, Greenbank, and Deysher (1927) have called "moloxides." They are alleged to be responsible for oxidation even *in vacuo*.

SUMMARY

The petroleum ether soluble matter of fresh and aged, canned orange juice has been investigated. In fresh juice this consists of unidentified waxy bodies insoluble in petroleum ether; oleic, linoleic, cerotic, palmitic, and stearic acids; unidentified aliphatic alcohols and resins; phytosterols; sterols; and carotinoid pigments.

The characteristics of the petroleum ether extract of aged, canned orange juice indicated that the fatty material had undergone oxidative changes, with the creation of hydroxy acids and other decomposition products, and had become rancid.

In view of the evidence presented it is believed that off-flavors subsequently developing in canned orange juice are, to some extent, due to the oxidation of the fat in the juice.

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STUDIES OF WESTERN TOMATOES

G. S. BOHART
National Canners Association, Western Branch Laboratories, San Francisco, California

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The desirable features of foods, including tomatoes, are related very largely to chemical composition. Considerable work has been done by various investigators on the chemistry of tomatoes, but the relationships between the various chemical constituents and the physical characteristics are still little known. It is known that the total solids content of tomatoes from different localities varies considerably, and in some instances the pH of tomatoes has been so high that sterilization without excessive tissue breakdown has been a difficult problem. More information is needed to understand the important factors which determine why some tomatoes break down more than others during cooking, and why color and flavor are variable even in the same variety of tomatoes grown in different fields.

It has been recognized for a long time that the composition of tomatoes is subject to both local and seasonal variations, but the extent to which this is true has not generally been appreciated. From the points of view both of nutritive value and of quality in flavor and color it is desirable to extend our knowledge of naturally occurring variations and of the manners in which various constituents and characteristics are related, as a basis for subsequent study of quality-control projects.

Much progress in the study of tomatoes has been made by numerous investigators, both by growing tomatoes in various nutrient solutions and by varying nutrient applications to soil plots. In both of these methods, variables are limited, for the most part, to nutrient materials. The present correlation study, comprehending as it does a relatively large growing area, represents another method of approach to the problem of quality studies.

COLLECTION AND PREPARATION OF SAMPLES

During the 1935 and 1936 canning seasons, samples of well-matured tomatoes were collected from 76 separate fields throughout California, Utah, and Colorado. In 1937, 65 additional samples were obtained, most of which were from fields not included in the 76 lots collected earlier. The fruit collected during the 1935 and 1936 seasons was selected on the basis of normal red color only, whereas all

lots included in the 1937 series were carefully sorted for solid-pack quality. In some instances less than ten per cent of the tomatoes examined were found suitable for the purpose. This rigid grading may account for the lack of closer agreement in some of the correlation values shown later (Tables 3 to 6 inclusive). In this work 12 varieties were included: Early Santa Clara, Stone, Earliana, Baltimore, Landreth, Rutgers, No. 133-6, No. 133-8, Cal 65, Prichard, Jack o' Hearts, and San Marzano (a pear-shaped tomato).

From each of the 76 samples collected during 1935 and 1936, approximately two kilograms of ripe fruit were immersed in boiling water for about 30 seconds. The tomatoes were then peeled, cored, ground through a household grinder, and thoroughly mixed by stirring; and, after a portion for pH, pectin, protopectin, and total acidity determinations had been set aside, a sample of suitable size for further analysis was placed in a glass-covered Mason jar. One milliliter of a 40-per cent formaldehyde solution per 100 grams of ground product was added as a preservative.

The 65 samples obtained in 1937 were selected from cannery stock representative of solid-pack tomatoes and cooked in No. 2½ cans, using commercial canning methods. A composite of three of these cans from each lot was used for the subsequent analytical study.

CONSTITUENTS STUDIED AND METHODS OF ANALYSIS

For convenience the factors studied may be somewhat arbitrarily grouped as follows: physical characteristics, general chemical constituents, and ash constituents.

Physical Characteristics

Total Solids: Determined by the tentative method for total solids in tomato products, *Methods of Analysis*, A.O.A.C. (1935) 499, except that the bulk of the moisture was removed by placing the dishes in a flat-bottomed, enamelled pan, resting on a steam bath. The samples were closely watched and were removed as soon as apparent dryness had been reached.

Insoluble Solids: Samples of filtrate were dried as under "Total Solids." The insoluble-solids content was then calculated by the formula:

$$I = \frac{100\ (T - S_o)^1}{100 - S_o}$$

where I represents the per cent of insoluble solids, T the per cent of total solids, and S_o the per cent of soluble solids in the filtrate.

[1] This formula was derived independently by W. F. Eberz in this laboratory.

Soluble Solids: Per cent of total solids less per cent of insoluble solids.

Alcohol-Insoluble Solids in Filtrate: The filtrate from 10 ml. of juice was precipitated with 100 ml. alcohol, filtered, and dried overnight at 100°C.(212°F.). The residue was then cooled and weighed.

Pulp: The term "pulp" as used in this work refers to insoluble solids other than seeds. Total solids other than seeds were determined by drying samples of tomatoes from which seeds had been screened. The true value for pulp was then calculated by the formula given under "Insoluble Solids" except that in this case "T" refers to the percentage of total solids of the strained whole tomatoes.

Seeds: A weighed sample of the whole product was screened and the seeds were then washed in water by decantation and dried as under "Total Solids."

Pigment: Determined on a chloroform extract in the Cenco-Sheard-Sanford Photelometer according to the following method developed in this laboratory:

Weigh out approximately 50 grams of the stock tomato sample. Remove seeds by screening. Pump the screened material through a hand homogenizer three successive times. Weigh five grams of the homogenized pulp in a sugar scoop. Transfer the weighed pulp to a four-ounce oil sample bottle and add water until the bottle is approximately half full. Pipette exactly 25 ml. of chloroform into the bottle, stopper, and shake for two minutes. Centrifuge until the layers separate. If separation does not occur promptly, invert the bottle gently several times and centrifuge again. Pipette 10 ml. from the clear chloroform extract and filter through a dry seven-cm. filter paper into a 50-ml. volumetric flask. Wash filter thoroughly and make up to volume with pure chloroform. Transfer to the one-cm. photelometer cell and read using a blue filter (Cenco No. 1).

Calibrate the photelometer using solutions of lycopene in chloroform of known concentration, and express results for total pigment as parts per million of lycopene. The lycopene was prepared from tomato paste by a method originating in this laboratory and upon which further work is anticipated. The pigments were extracted with chloroform, the chloroform solution evaporated to dryness, and the dry residue extracted with acetone to remove pigments other than lycopene. Final purification was effected by crystallization from a hot acetone solution.

General Chemical Constituents

Total Acidity: Total acidity was determined by titrating to a pH of 7 with brom-thymol blue indicator. The results are expressed as per cent of hydrated citric acid ($C_6H_8O_7 \cdot H_2O$).

pH: The pH was determined by means of the hydrogen point electrode, using the method of Ehrenberg (1929).

Ash: The sample was heated in a muffle at 500°C.(932°F.), using a platinum dish.

At approximately two-hour intervals the charred cake was extracted with water and the insoluble material returned to the furnace until finally all carbon had disappeared. Soluble and insoluble ash materials were then combined, evaporated, heated to 500°C. for five minutes, cooled in a desiccator, and weighed.

Total Sugars: Determined as invert sugar, employing the Munson and Walker General Method, as given in *Methods of Analysis*, A.O.A.C. (1935) XXXIV, **18(b)**, **18(d)**, **23(c)**, **36**, **37**, and **38**, pages 468-469, 473, 479-480. Results are expressed as per cent of total sugars, calculated as invert sugar.

Protein: Nitrogen was determined by the Kjeldahl-Gunning-Arnold method for "Organic and Ammoniacal Nitrogen Only," as given in *Methods of Analysis*, A.O.A.C. (1935) II, **25**, p. 25. The value for nitrogen content was multiplied by the factor 6.25, and the result expressed as per cent of protein (N x 6.25).

Pectin: Determined by the method of Dorée (1933), and the result expressed as per cent of pectic acid.

Protopectin: Determined by the method of Dorée (1933), and the result expressed as per cent of pectic acid.

Crude Fiber: Estimated according to the method given by *Methods of Analysis*, A.O.A.C. (1935) XXVII, **25**, **26**, **27**, pages 340, 341, with the following deviations from the stated procedure: (a) Digestion was carried out in a 400-ml. beaker, giving a depth of liquid of 1⅞ inches in place of the 1½-inch maximum called for in the official statement. (b) Handkerchief linen which has a smooth surface was used for filtration of the samples. After each of the three filtrations the sample was removed from the cloth by means of a stainless steel spatula, in place of the washing procedure called for in the method. (c) The final washing of the sample with 95 per cent alcohol, as called for, was omitted.

Ash Constituents

Potassium: Double precipitation as $KClO_4$ from a solution of the ash was employed for 1935 and 1936 samples, Treadwell-Hall (1934). In the 1937 series potassium was determined by the cobaltinitrite precipitation procedure of Wilcox (1937), with the following modifications: Oxidation of the precipitated potassium cobaltinitrite was carried out with hot excess standard (.05 N) ceric sulphate

solution, the excess being titrated with standard ferrous sulphate solution, using ortho-phenanthroline ferrous sulphate as indicator.

Calcium: Precipitated as calcium oxalate and titrated with potassium permanganate solution, according to the tentative procedure given by *Methods of Analysis*, A.O.A.C. (1935) XXVI, **19**, p. 323.

Magnesium: Determined gravimetrically as $Mg_2P_2O_7$, according to the method given by *Methods of Analysis*, A.O.A.C. (1935) XXVI, **20**, p. 323-324.

Iron: Precipitated as ferric phosphate according to the method given for removal of iron preparatory to determination of manganese. See *Methods of Analysis*, A.O.A.C. (1935) XXVI, **17**, p. 322. A sulfuric acid solution of the phosphate was treated according to the colorimetric procedure given by Scott (1925), p. 261. In place of the Nessler tubes suggested in this procedure, however, the iron was determined colorimetrically in a Cenco-Sheard-Sanford Photelometer.

Manganese: Converted to $KMnO_4$ according to *Methods of Analysis*, A.O.A.C. (1935) XXVI, **17**, p. 322. Potassium permanganate was determined colorimetrically in a Cenco-Sheard-Sanford Photelometer.

Copper: Determined by a method based upon the Winter (1936) modification of the Haddock-Evers Method. The method employed was as follows:

Weigh out a 50-gram sample into a 100-mm. porcelain dish possessing a good glazed surface. Add five ml. of "ash aid,"[2] mix thoroughly, and evaporate to dryness on water bath. Heat on a hot plate or in a muffle at a low temperature, 200-300°C. (392-572°F.), until sample is thoroughly charred, then continue heating at 500°C. (932°F.) until further ashing becomes very slow (usually from three to five hours is sufficient). Remove from muffle, cool, add water and .5 ml. of "ash aid," and mix thoroughly with a stirring rod, breaking up any hard lumps of carbon which may remain. Dry *thoroughly* on water bath and replace in muffle at 500°C. for 15 to 30 minutes. If more than a trace of carbon remains at this stage, repeat leaching procedure using a few ml. of water and two to three drops of "ash aid," being careful to break up any lumps of carbon remaining.

To the ash add 10 ml. of an approximate 24-per cent solution of HBr and 10 ml. of HCl (one volume of concentrated HCl made up to two volumes with water) both reagents having been distilled in pyrex glass. Evaporate on water bath, swirling the dish occasion-

[2] *Ash aid solution:* Dissolve 40 grams of $Al(NO_3)_3 \cdot 9H_2O$ and 20 grams of $Ca(NO_3)_2 \cdot 4H_2O$ in 100 ml. of water. Add .2-.5 gram filter cel and saturate the solution with H_2S. Warm, filter, and boil to expel H_2S. Adjust to original volume (about 130 ml.).

ally to ensure complete extraction of copper both from the ash and from the surface of the dish. After the solution has reached approximate dryness, add 10 ml. of the dilute HCl and a few ml. of water. Warm until ash is dissolved and filter into a 50-ml. volumetric flask, washing the paper thoroughly with hot water. Make up to volume and mix thoroughly.

Transfer a 20-ml. aliquot from the volumetric flask to a 250-ml. separatory funnel marked to indicate a volume of 110 ml. (The aliquot taken should contain not more than 50 micrograms of copper.) Add 20 ml. of citric acid solution [3] and mix. Add four drops of cresol red indicator and concentrated NH_4OH until the first appearance of red, then add .4 ml. in excess. Add 20 ml. of a .1-per cent solution of sodium diethyldithiocarbamate and make up with water to the 110-ml. calibration mark. Finally add 20 ml. of carbon tetrachloride *accurately measured* and shake vigorously for about one minute. Dry the stem of the funnel with a cotton applicator, then insert a loose cotton plug approximately one inch in length. Draw off the CCl_4 solution into the dry one-cm. photelometer cell and read, using a blue filter (Cenco No. 1).

Prepare a calibration curve for the photelometer using a standard copper solution (one ml. contains one microgram of copper). The curve should cover the range of 0 to 50 micrograms of copper.

Blank determinations should be run at frequent intervals as a check on the reagents used.

The blank is deducted from the observed reading for any sample.

Phosphorus: Determined in the 1935 and 1936 samples by the ammonium phosphomolybdate precipitation and subsequent titration, according to *Methods of Analysis*, A.O.A.C. (1935) II, **12**, p. 21. In the 1937 series the molybdenum-blue colorimetric method of Zinzadze (1935) was employed in conjunction with the wet-digestion method of Gerritz (1935) for preparation of the samples.

Sulfur: Determined by the method given in *Methods of Analysis*, A.O.A.C. (1935) XXVI, **11**, p. 321.

Chlorine: Determined by the method given in *Methods of Analysis*, A.O.A.C. (1935) XII, **35**, p. 131.

ANALYTICAL DATA

Maximum, minimum, and average values of analyses for all varieties and localities investigated are shown (Table 1). Analytical results for tomatoes of the San Marzano variety, indicate that the

[3] ***Citric acid solution:*** Dissolve 150 grams of citric acid in 700 ml. of water, add 100 ml. of concentrated NH_4OH, add filter eel, and saturate with H_2S. Warm, filter, boil off H_2S, cool, and dilute to 1,000 ml.

amounts of most of the constituents, except pulp, insoluble solids, calcium, and total acidity, are of the same order of magnitude as were found for the same constituents in the composite of analyses for other varieties of tomatoes. In the case of insoluble solids, the difference is largely due to the pulp constituent.

A comparison of the ratio of iron to manganese (Table 1) shows that, with the exception of the maximum value for the San Marzano variety, the percentage of iron is consistently in the neighborhood of ten times that of manganese.

RELATIVE PROPORTIONS OF FLESH AND OF FREE LOCULE CONTENTS

The separation of flesh from free locule contents was accomplished as follows: After peeling with the aid of boiling water, each tomato was cut in two parts at the equator. The contents of the seed cells, consisting of jelly-like pulp and seeds, were then scraped out with the handle of a slender spoon leaving most of the "inner locule pulp"[4] attached to the outer walls. The flesh and the "inner locule pulp" were preserved together for analysis and designated "flesh." The jelly-like pulp and seeds of the locule were designated "free locule contents."

The relative proportions of flesh and of free locule contents were as follows:

Sample No.	Variety	Whole tomato	
		Flesh	Free locule contents
		pct.	*pct.*
1	Stone	68.4	31.6
2	Stone	71.2	28.8
3	Early Santa Clara	81.3	18.7
4	San Marzano[1]	83.4	16.6

[1] Pear shaped.

Results of separate analyses of the flesh and of the free locule contents, including seeds, of four lots of tomatoes are shown (Table 2), extending work previously reported by Bigelow (1917), Bigelow and Stevenson (1923), MacGillivray and Ford (1928), and Saywell and Cruess (1932).

Samples 1 and 2 represent the Stone variety from two localities about ten miles apart. No. 3, an early Santa Clara tomato, came from a third locality, and No. 4 was of the San Marzano variety

[4] So designated by MacGillivray and Ford (1928).

TABLE 1

Composition of Western Tomatoes

Constituent (or factor)	Varieties other than San Marzano [1]				San Marzano [1] variety			
	Number of samples analyzed	Maximum	Minimum	Average	Number of samples analyzed	Maximum	Minimum	Average
pH	136	4.61	4.08	4.38	5	4.62	4.33	4.44
		pct.	*pct.*	*pct.*		*pct.*	*pct.*	*pct.*
Total solids	136	9.13	5.38	7.11	5	8.20	7.03	7.52
Insoluble solids	136	1.61	0.71	1.10	5	1.78	1.25	1.51
Soluble solids	136	7.52	4.47	6.01	5	6.61	5.78	6.02
Alcohol-insoluble solids in filtrate	65	0.48	0.16	0.27	....	....	....	....
Pulp	113	0.95	0.50	0.67	5	1.16	0.87	1.01
Seeds	113	0.89	0.28	0.45	5	0.62	0.38	0.50
Crude fiber	29	0.31	0.20	0.27	....	....	....	....
Total acidity, as citric [2]	136	0.62	0.24	0.36	5	0.30	0.24	0.28
Ash	71	0.75	0.39	0.55	5	0.58	0.54	0.56
Total sugars, as invert	48	5.36	2.57	3.76	....	....	....	....
Protein (N x 6.25)	136	1.37	0.59	0.92	5	1.14	0.66	0.78
Pectin, as pectic acid	65	0.46	0.03	0.14	....	....	....	....
Protopectin, as pectic acid	65	0.15	0.02	0.08	....	....	....	....
		p.p.m.	*p.p.m.*	*p.p.m.*		*p.p.m.*	*p.p.m.*	*p.p.m.*
Pigment, as lycopene	65	140	72	104	....	....	....	....
Potassium	70	3,200	1,640	2,410	5	2,530	2,270	2,420
Calcium	71	152	24	59	5	131	54	78
Magnesium	71	158	75	121	5	128	92	115
Iron	60	10.8	2.8	5.9	....	11.1	4.7	6.6
Manganese	60	1.1	.2	.6	....	.7	.5	.6
Copper	44	2.3	.6	1.5	5	2.6	1.0	1.8
Phosphorus	71	309	112	234	5	305	190	232
Sulfur	71	115	54	82	5	108	74	84
Chlorine	71	728	138	409	5	528	211	420

[1] Tomatoes of the San Marzano variety are of the pear-shaped type. [2] Hydrated citricacid ($C_6H_8O_7 \cdot H_2O$).

TABLE 2

Composition of Flesh and of Free Locule Contents of Tomatoes

Tomato sample No.	Flesh					Free Locule Contents				
	1	2	3	4	Av.	1	2	3	4	Av.
Variety	Stone	Stone	Early Santa Clara	San Marzano [1]		Stone	Stone	Early Santa Clara	San Marzano [1]	
	pct.	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*
Total solids	5.79	6.46	8.15	7.53	6.98	6.82	7.03	8.73	7.98	7.64
Total solids other than seeds	5.74	6.39	8.08	7.45	6.91	5.54	5.97	7.34	6.33	6.29
Soluble solids	5.03	5.66	7.25	6.26	6.05	5.17	5.62	6.89	5.77	5.86
Insoluble solids	.76	.80	.90	1.27	.93	1.65	1.41	1.84	2.21	1.78
Pulp	.71	.73	.83	1.19	.86	.37	.35	.45	.56	.43
Seeds	.05 [2]	.07 [2]	.07 [2]	.08 [2]	.07 [2]	1.28	1.06	1.39	1.65	1.34
Ash	.56	.43	.46	.56	.50	.64	.58	.59	.73	.63
Total sugars, as invert	2.92	4.13	5.42	4.47	4.23	2.56	3.45	4.52	3.33	3.46
Protein (N x 6.25)	.78	.59	.70	.57	.66	1.35	1.03	1.32	1.29	1.25
Total acidity, as citric [3]	.36	.21	.25	.27	.27	.70	.55	.42	.45	.53
pH	4.40	4.47	4.46	4.30	4.41	4.17	4.24	4.46	4.46	4.33
	p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*
Potassium	2,570	1,880	2,470	2,240	2,240	2,770	2,290	2,310	2,910	2,570
Calcium	52	51	40	85	57	62	50	44	71	56
Magnesium	127	114	84	93	104	183	148	188	217	169
Iron	7.6	4.8	4.3	6.1	5.7	10.8	6.3	10.5	3.4	7.8
Manganese	.6	.2	.2	.1	.3	2.1	1.2	1.4	1.8	1.6
Copper	.9	.6	1.8	1.6	1.2	2.0	1.2	2.2	1.8	1.8
Phosphorus	134	169	188	223	178	265	362	373	518	379
Sulfur	67	50	95	105	79	78	69	108	123	105
Chlorine	502	356	331	555	436	386	268	308	412	343

[1] Pear shaped. [2] Had the mechanical separation been perfect, this value would have been zero. [3] Hydrated citric acid ($C_6H_8O_7 \cdot H_2O$).

from a region approximately 60 miles from the field in which Sample 1 was grown.

It will be seen that in general the percentage of ash constituents in the free locule contents is higher than in the flesh portion. This is to be expected since the seeds were included with the free locule contents and it is well known that mineral constituents are concentrated in the seeds. The relatively high proportion of both total acid and ash in the free locule contents is in line with the close correlation between these two factors (Tables 3 to 6 inclusive).

In view of the fact that the total acidity is higher in the free locule contents than in the flesh of the tomato, it is to be expected that fruit with relatively large locules would tend to be more acid

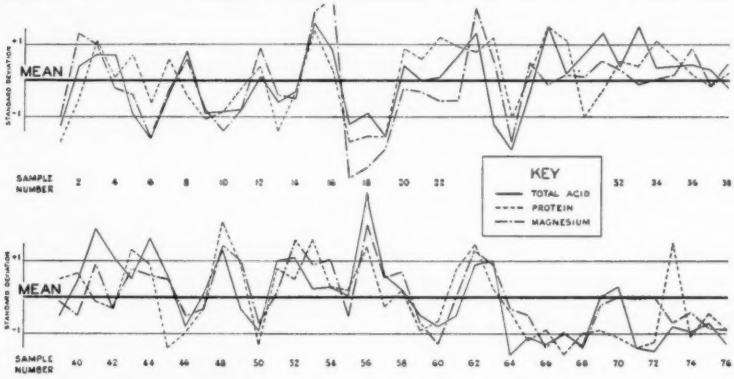

FIG. 1. Relationship between total acidity, protein, and magnesium based on deviations from the mean.

Note: Individual values are represented on the basis of the individual deviations from the mean, expressed as a percentage of the standard deviation. The term "mean" denotes the average of all 76 samples.

than that in which the proportion of flesh is large; this is borne out by results (Table 2).

Particularly noteworthy is the distribution of chlorides. On the basis of the four samples, analytical results of which are shown (Table 2), the percentage of chlorides, unlike other ash constituents, is higher in the flesh portion of the tomato than in the free locule contents.

CORRELATION OF ANALYTICAL RESULTS

A preliminary inspection of analytical results obtained on tomatoes indicated that reasonably close correlations existed between the percentages of certain of the constituents. In order to illustrate some of the more significant relationships the results have been plotted (Fig. 1) in such a way that the average of all 76 samples for the

constituents of total acidity, protein, and magnesium coincide. By applying proper factors to the data the scales for plotting the standard deviations of these three constituents have also been made to coincide.

The high degree of correlation shown (Fig. 1) suggested the advisability of making further computations. Correlation coefficients, using the method given by Snedecor (1937), have been calculated for all combinations of all constituents shown (Tables 3 to 6 inclusive). Highly significant values are shown in boldface type and significant values are shown in italics. Blank spaces indicate that the correlations for those combinations have been computed but were found to be not significant. A minus sign preceding a significant symbol indicates significant correlation in the negative sense; for example, in Table 3 under "Seeds," it will be seen that the value for "Sugar" is given as —.38 which means that the higher the percentage of seeds found in the tomatoes analyzed, the lower the percentage of sugar.

Significant correlations of experimental results where nutrient supplies constitute the only variable apply for a particular climate and nutrient supply (aqueous or soil) but may not necessarily apply for other climatic and soil conditions. Where a significant correlation is found between any two constituents for tomatoes grown under widely varying conditions of climate, soil, and nutrient supply, however, the findings so obtained constitute a basis for a relatively broad generalization functional under natural conditions.

The data show correlation results on the ordinary wet basis of the edible portion of tomatoes (Tables 3 and 5), and correlation values with the total solids variable eliminated (Tables 4 and 6). The only variable eliminated by partial correlation in this work is total solids. Since there may be interrelationships between any of the other constituents, a complete picture could be obtained only by repeating partial correlation calculations with each of the other factors eliminated separately. Because of the great amount of time that would be involved, this has not been done except in the case of total solids, where the adjustment would appear to be more significant than with regard to any other factor studied.

The significance of any particular value of correlation coefficient is dependent upon the number of samples represented. The number of samples involved (Tables 3 and 4) was 71 with the exception of 60 for iron and manganese and 51 for pulp, seeds, and sugar. The data given (Tables 5 and 6) included 65 samples with the following exceptions: phosphorus, 27; crude fiber, 29; potassium, magnesium, and calcium, 39. The range of correlation coefficients for various numbers of samples according to Snedecor (1937) is shown (Table 7).

TABLE 3

Correlation Coefficients[1] for Various Constituents of Western Tomatoes on Wet Basis—1935 and 1936 Seasons

Constituents	Total solids	Soluble solids	Insoluble solids	Seeds	Pulp	Total sugars	Ash	Protein (N x 6.25)	Total acidity	H+ concentration	Magnesium	Calcium	Potassium	Chlorine	Phosphorus	Iron	Sulfur	Manganese
Total solids	....[2]	**.94**	**.41**	....	**.53**	**.83**	....	....	....	....	....	....	....	....	....	....	....	....
Soluble solids	**.94**	....	....	....	**.39**	**.86**	....	....	....	....	....	....	....	....	....	....	....	....
Insoluble solids	**.41**	....	....	**.66**	**.70**	....	**.39**	*.30*	**.31**	....	**.39**	**.63**	....	**.33**	....	*.39*	*.28*	....
Seeds	....	....	**.66**	....	....	**—.38**	**.54**	**.37**	**.64**	**.38**	**.54**	**.48**	*.35*	**.37**	....	**.41**	*.35*	....
Pulp	**.53**	**.39**	**.70**	....	....	**.42**	....	....	**—.41**	*—.29*	....	*.29*	....	....	....	....	....	....
Total sugars	**.83**	**.86**	....	**—.38**	**.42**	....	**—.41**	....	**—.49**	*—.31*	*—.33*	....	*—.29*	**—.37**	....	....	....	*—.33*
Ash	....	....	**.39**	**.54**	....	**—.41**	....	**.40**	**.61**	....	**.52**	**.31**	**.89**	**.49**	**.49**	....	**.46**	....
Protein (N x 6.25)	....	....	*.30*	**.37**	....	....	**.40**	....	**.63**	....	**.64**	**.33**	*.27*	*.26*	....	**.44**	**.34**	**.34**
Total acidity	....	....	**.31**	**.64**	**—.41**	**—.49**	**.61**	**.63**	....	**.57**	**.73**	**.33**	**.44**	**.30**	*.25*	....	*.24*	*.29*
H+ concentration	....	....	....	**.38**	*—.29*	*—.31*	....	....	**.57**	....	**.36**	....	*—.24*	....	....	....	....	....
Magnesium	....	....	**.39**	**.54**	....	*—.33*	**.52**	**.64**	**.73**	**.36**	....	**.37**	**.32**	**.35**	*.26*	**.42**	*.28*	**.36**
Calcium	....	....	**.63**	**.48**	*.29*	....	**.31**	**.33**	**.33**	....	**.37**	....	....	....	....	*.31*	**.34**	....
Potassium	....	....	....	*.35*	....	*—.29*	**.89**	*.27*	**.44**	*—.24*	**.32**	....	....	**.37**	**.48**	....	**.31**	....
Chlorine	....	....	**.33**	**.37**	....	**—.37**	**.49**	*.26*	**.30**	....	**.35**	....	**.37**	....	....	....	*.28*	....
Phosphorus	....	....	....	....	....	....	**.49**	....	*.35*	....	*.26*	....	**.48**	....	....	....	....	....
Iron	....	....	*.29*	**.41**	....	....	....	**.44**	....	....	**.42**	*.31*	....	....	....	....	....	**.37**
Sulfur	....	....	*.28*	*.35*	....	....	**.46**	**.34**	*.24*	....	*.28*	**.34**	**.31**	*.28*	....	....	....	....
Manganese	....	....	....	....	....	*—.33*	....	**.34**	*.29*	....	**.36**	....	....	....	....	**.37**	....	....

[1] "Highly significant" values are shown in boldface type; "significant" values are shown in italics. [2] Blank spaces in all cases indicate that the correlation coefficient was computed and found to be not significant.

TABLE 4

Correlation Coefficients[1] for Various Constituents of Western Tomatoes With Total Solids Variable Eliminated by Partial Correlation—1935 and 1936 Seasons

Constituents	Soluble solids	Insoluble solids	Seeds	Pulp	Total sugars	Ash	Protein (N x 6.25)	Total acidity	H+ concentration	Magnesium	Calcium	Potassium	Chlorine	Phosphorus	Iron	Sulfur	Manganese
Soluble solids	....[2]	**—.84**	**—.52**	**—.58**	**.41**	**—.37**	....	*—.23*	....	*—.30*	**—.62**	....	**—.36**	....	....	....	....
Insoluble solids	**—.84**	....	**.71**	**.62**	**—.60**	**.40**	*.26*	**.34**	*.29*	**.38**	**.68**	....	**.41**	....	*.32*	....	....
Seeds	**—.52**	**.71**	....	....	**—.74**	**.54**	**.37**	**.64**	**.39**	**.54**	**.48**	*.35*	**.38**	....	**.41**	*.35*	....
Pulp	**—.58**	**.62**	....	....	....	....	....	**—.47**	....	....	*.33*	....	....	*—.33*	....	....	....
Total sugars	**.41**	**—.60**	**—.74**	....	....	**—.82**	**—.72**	**—.87**	*—.35*	**—.74**	**—.49**	**—.56**	**—.51**	....	....	**—.55**	....
Ash	**—.37**	**.40**	**.54**	....	**—.82**	....	**.40**	**.62**	....	**.52**	**.31**	**.89**	**.50**	**.49**	....	**.46**	....
Protein (N x 6.25)	....	*.26*	**.37**	....	**—.72**	**.40**	....	**.64**	....	**.64**	**.33**	*.27*	*.28*	....	**.45**	**.32**	**.38**
Total acidity	*—.23*	**.34**	**.64**	**—.47**	**—.87**	**.62**	**.64**	....	**.57**	**.74**	**.33**	**.44**	**.31**	*.25*	....	*.24*	*.29*
H+ concentration	....	*.29*	**.39**	....	*—.35*	....	....	**.57**	....	**.38**	....	*—.24*	....	....	....	....	....
Magnesium	*—.30*	**.38**	**.54**	....	**—.74**	**.52**	**.64**	**.74**	**.38**	....	**.37**	**.32**	**.36**	*.26*	**.42**	*.26*	**.39**
Calcium	**—.62**	**.68**	**.48**	*.33*	**—.49**	**.31**	**.33**	**.33**	....	**.37**	....	....	....	....	*.31*	**.35**	....
Potassium	....	....	*.35*	....	**—.56**	**.89**	*.27*	**.44**	*—.24*	**.32**	....	....	**.37**	**.48**	....	**.31**	....
Chlorine	**—.36**	**.41**	**.38**	....	**—.51**	**.50**	*.28*	**.31**	....	**.36**	....	**.37**	....	....	....	**.32**	....
Phosphorus	....	....	....	*—.33*	....	**.49**	....	*.25*	....	*.26*	....	**.48**	....	....	....	....	....
Iron	....	*.32*	**.41**	....	....	....	**.45**	....	....	**.42**	*.31*	....	....	....	....	....	**.38**
Sulfur	....	....	*.35*	....	**—.55**	**.46**	**.32**	*.24*	....	*.26*	**.35**	**.31**	**.32**	....	....	....	....
Manganese	....	....	....	....	....	....	**.38**	*.29*	....	**.39**	....	....	....	....	**.38**	....	....

[1] "Highly significant" values are shown in boldface type; "significant" values are shown in italics. [2] Blank spaces in all cases indicate that the correlation coefficient was computed and found to be not significant.

TABLE 5

Correlation Coefficients[1] for Various Constituents of Western Tomatoes on Wet Basis—1937 Season

Constituents	Total solids	Soluble solids	Insoluble solids	Seeds	Pulp	Total sugars	Protein (N x 6.25)	Crude fiber	Total acidity	H+ concentration	Magnesium	Calcium	Potassium	Phosphorus[2]	Manganese	Alcohol-insoluble solids in filtrate	Pectin, as pectic acid	Protopectin, as pectic acid	Pigment, as lycopene
Total solids	....[2]	**.97**	**.75**	**.46**	**.73**	**.81**	....	*.38*	**.56**	**.32**	....	*.33*	....	....	....	**.44**	**.40**	....	....
Soluble solids	**.97**	....	**.60**	**.36**	**.66**	**.83**	....	....	**.51**	....	....	....	....	....	....	**.48**	**.39**	....	....
Insoluble solids	**.75**	**.60**	....	**.73**	**.86**	**.61**	*.33*	*.44*	**.45**	**.56**	*.33*	**.61**	....	....	....	....	**.34**	....	....
Seeds	**.46**	**.36**	**.73**	....	*.31*	**.39**	*.28*	....	**.35**	**.46**	....	....	**—.44**	....	....	....	....	....	....
Pulp	**.73**	**.66**	**.86**	*.31*	....	**.55**	....	**.65**	**.46**	**.37**	....	**1.00**	....	....	....	*.28*	**.43**	**.32**	....
Total sugars	**.81**	**.83**	**.61**	**.39**	**.55**	....	....	....	....	....	....	**.66**	....	....	....	**.38**	**.34**	....	....
Protein (N x 6.25)	....	....	**.33**	*.28*	....	....	....	**—.48**	**.57**	*.26*	*.33*	*.32*	....	....	**.40**	....	....	....	**.32**
Crude fiber	*.38*	....	*.44*	....	**.65**	....	**—.48**	....	....	....	....	....	....	....	....	*.39*	*.37*	....	**—.56**
Total acidity	**.56**	**.51**	**.45**	**.35**	**.46**	....	**.57**	....	....	**.50**	**.52**	**.43**	....	....	**.33**	....	....	....	....
H+ concentration	**.32**	....	**.56**	**.46**	**.37**	....	*.26*	....	**.50**	....	*.40*	....	**—.50**	....	....	....	*.26*	....	....
Magnesium	....	....	*.33*	....	....	....	*.33*	....	**.52**	*.40*	....	....	....	....	*.35*	....	....	....	....
Calcium	*.33*	....	**.61**	**1.00**	**.66**	....	*.32*	....	**.43**	....	....	....	....	....	....	....	**....**	....	....
Potassium	....	....	....	**—.44**	....	....	....	....	....	**—.50**	....	....	....	....	....	*.37*	*—.34*	....	....
Phosphorus[2]	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
Manganese	....	....	....	....	....	....	**.40**	....	**.33**	....	*.35*	....	....	....	....	**—.39**	....	*—.25*	....
Alcohol-insoluble solids in filtrate	**.44**	**.48**	....	....	*.28*	**.38**	....	*.39*	....	....	....	....	*.37*	....	**—.39**	....	....	**.39**	....
Pectin, as pectic acid	**.40**	**.39**	**.34**	....	**.43**	**.34**	....	*.37*	....	*.26*	....	....	*—.34*	....	....	....	....	....	....
Protopectin, as pectic acid	....	....	....	....	**.32**	....	....	....	....	....	....	....	....	....	*—.25*	**.39**	....	....	....
Pigment, as lycopene	....	....	....	....	....	....	**.32**	**—.56**	....	....	....	....	....	....	....	....	....	....	....

[1] "Highly significant" values are shown in boldface type; "significant" values are shown in italics. [2] Blank spaces in all cases indicate that the correlation coefficient was computed and found to be not significant.

TABLE 6

Correlation Coefficients[1] for Various Constituents of Western Tomatoes With Total Solids Variable Eliminated by Partial Correlation—1937 Season

Constituents	Soluble solids	Insoluble solids	Seeds	Pulp	Total sugars	Protein (N x 6.25)	Crude fiber	Total acidity	H+ concentration	Magnesium	Calcium	Potassium	Phosphorus[2]	Manganese	Alcohol-insoluble solids in filtrate	Pectin, as pectic acid	Protopectin, as pectic acid	Pigment, as lycopene
Soluble solids	....[2]	**—.86**	**—.43**	*—.30*	**.33**	....	*—.43*	....	**—.53**	....	**—.52**	....	....	....	....	....	....	....
Insoluble solids	**—.86**	....	**.65**	**.68**	....	....	....	....	**.51**	....	**.59**	....	....	....	....	**.34**	....	**—.38**
Seeds	**—.43**	**.65**	....	....	....	**.33**	....	....	**.37**	....	**1.00**	**—.55**	....	....	....	....	....	....
Pulp	*—.30*	**.68**	....	....	....	....	**.57**	....	....	....	**.65**	....	....	....	....	....	....	**—.43**
Total sugars	**.33**	....	....	....	....	**—.51**	....	**—.45**	....	....	....	....	....	....	....	....	....	....
Protein (N x 6.25)	....	....	**.33**	....	**—.51**	....	**—.63**	**.68**	....	....	....	....	....	**.37**	*—.31*	....	....	*.31*
Crude fiber	*—.43*	....	....	**.57**	....	**—.63**	....	....	....	....	....	....	....	....	....	....	....	**—.66**
Total acidity	....	....	....	....	**—.45**	**.68**	....	....	**.41**	**.44**	....	....	....	*.28*	....	....	....	....
H+ concentration	**—.53**	**.51**	*.37*	....	....	....	....	**.41**	....	*.34*	....	**—.57**	....	....	*—.29*	....	....	....
Magnesium	....	....	....	....	....	....	....	**.44**	*.34*	....	....	....	....	....	....	....	....	....
Calcium	**—.52**	**.59**	**1.00**	**.65**	....	....	....	....	....	....	....	....	....	....	*—.40*	....	....	....
Potassium	....	....	**—.55**	....	....	....	....	....	**—.57**	....	....	....	....	....	....	*.36*	**—.42**	....
Phosphorus[2]	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
Manganese	....	....	....	....	....	**.37**	....	*.28*	....	....	....	....	....	....	**—.54**	....	*—.30*	....
Alcohol-insoluble solids in filtrate	....	....	....	....	....	*—.31*	....	....	*—.29*	....	*—.40*	*.36*	....	**—.54**	....	....	**.33**	....
Pectin, as pectic acid	....	**.34**	....	....	....	....	....	....	....	....	....	**—.42**	....	....	....	....	....	....
Protopectin, as pectic acid	....	....	....	....	....	....	....	....	....	....	....	....	....	*—.30*	**.33**	....	....	....
Pigment, as lycopene	....	**—.38**	....	**—.43**	....	*.31*	**—.66**	....	....	....	....	....	....	....	....	....	....	....

[1] "Highly significant" values are shown in boldface type; "significant" values are shown in italics. [2] Blank spaces in all cases indicate that the correlation coefficient was computed and found to be not significant.

A comparison of the correlation coefficients found in different tables for the same pair of constituents makes it apparent that in most instances the values are in good agreement. In one case only where the total-solids variable has been eliminated are there significant symbols with opposite signs for a given pair of factors. Correlation results have been obtained separately on analyses of 25 samples of the Stone and 24 samples of the early Santa Clara varieties. These correlation values were found to be in close agreement with those obtained on the composite of different varieties shown (Tables 3 to 6 inclusive) and are, therefore, not included in this paper. It should be borne in mind that although the tomato samples analyzed in this investigation were red ripe, we have no record of the length of time

TABLE 7

Significance of Correlation Coefficients When Different Numbers of Samples Are Used

Number of samples	Range of correlation coefficients	
	Significant	Highly significant
27	.381-.487	.487-1.00
29	.367-.479	.470-1.00
39	.317-.408	.408-1.00
51	.274-.356	.356-1.00
60	.255-.331	.331-1.00
65	.245-.318	.318-1.00
71	.234-.304	.304-1.00

they had been ripe before picking. It is possible, therefore, that some of the data, such as pH and total acidity, reflect variations in maturity between samples.

SUMMARY AND CONCLUSIONS

During the interval 1935 to 1937, inclusive, 141 samples of tomatoes, representing 12 varieties, were collected from various parts of California, Colorado, and Utah for an analytical study. Determinations included total solids, insoluble solids, soluble solids, alcohol-insoluble solids in filtrate, pulp, seeds, pigment, crude fiber, total acidity, pH, ash, sugar, protein, pectin, protopectin, potassium, calcium, magnesium, iron, manganese, copper, phosphorus, sulfur, and chlorine.

Correlation coefficients comprising practically all combinations of the analytical factors have been calculated and a large number of the chemical constituents and physical characteristics were found to have a highly significant correlation. Some interesting examples of consistent correlation are as follows:

Positive Correlations	*Negative Correlations*
Pulp and calcium	H+ concentration and potassium
Total acidity and magnesium	Sugar and ash
Total acidity and manganese	Sugar and total acidity
Total acidity and protein	Crude fiber and pigment
Total acidity and H+ concentration	
Seeds and H+ concentration	
Protein and manganese	
Magnesium and H+ concentration	
Insoluble solids and calcium	
Insoluble solids and ash	
Insoluble solids and magnesium	
Insoluble solids and chlorine	
Protein and ash	
Protein and sulfur	

The ratio between iron and manganese as regards high, low, and average results of 60 samples of raw tomatoes was found to be approximately one part of manganese to ten parts of iron.

The flesh and free locule contents of tomatoes collected from four separate localities were also analyzed.

In general, the percentage of ash constituents in the free locule contents was found to be higher than in the flesh portion. This is to be expected since the seeds were included with the free locule contents and it is well known that mineral constituents are concentrated in the seeds. The reverse was true in the case of chlorine.

In this investigation the fruit only was considered. Further work should be done on comparable analyses of other portions of the tomato plant and on the soil in which it is grown.

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ESTIMATION OF DECOMPOSITION OF FISH MUSCLE

H. C. BRADLEY AND B. E. BAILEY
Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin

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The problem of developing a satisfactory method for detecting the early stages of decomposition of flesh foods has received attention from chemists, and bacteriologists for many years. It appears, however, that there is no test which can replace reliably the simple organoleptic reactions of taste and smell. The development of spoilage is a complex phenomenon, and the reactions which produce a recognizable condition of spoilage in all probability differ with the species, the tissue, and the conditions. If a tissue were sterile and remained so, the processes of autolysis alone would produce changes which in time would constitute spoilage from a commercial standpoint because of the physical softening and alteration in taste. The presence of microörganisms introduces another set of variables which depends on the varieties present and the conditions of the environment. An objective test which could be used as a reliable criterion of the condition of a tissue must parallel the decomposition produced by autolysis alone and also by infective agents. It should measure autolytic changes and also the rapid increase of the products of bacterial metabolism.

One of the most delicate and characteristic tests used for measuring the primary cleavage in proteins is the development of a blue color by tyrosine, either free or in the peptide form, with the molybdic phosphoric reagent of Folin and Denis (1912b) and Folin and Ciocalteu (1927). This reaction was used by Bradley (1922) to measure autolysis. Its correlation with the primary cleavages in proteolysis was studied by Torbet and Bradley (1931). More recently Anson (1938) has used it in the estimation of pepsin, trypsin, and cathepsin. It is here called the tyrosine reaction merely for convenience. It measures the reactive tyrosine complexes and other phenols, tryptophane, cystine, sulfhydryl compounds including hydrogen sulfide, and other reducing agents. This makes the reagent peculiarly applicable to the detection of tissue spoilage, because a number of the compounds which are known to develop during storage will give the color. If other phenolic or reducing compounds are formed, the reaction will be intensified. For these reasons it was considered probable that this reaction would give a fairly close cor-

relation with the spoilage phenomena. In addition the studies of autolysis indicate that fresh normal tissues give a characteristically low tyrosine reaction.

In the following pages we wish to present studies of the disintegration of fish-muscle tissue as measured by this technique. In order to find out whether this reaction might be suitable for the purpose under consideration a preliminary study was made in which the changes in the free tyrosine content were compared with those in noncoagulable nitrogen and in pH, both of which have been used for measuring spoilage changes in flesh foods. For these preliminary experiments only a small number of fish were used. Subsequently larger numbers of fish were stored under various conditions and the increase in intensity of the reaction followed. The German carp *(Cyprinus carpio)*, the Pacific herring *(Clupea pallasii)*, and the pink or humpback salmon *(Oncorhynchus gorbuscha)* were studied in these experiments.

EXPERIMENTAL METHODS AND DATA

In the preliminary experiments, for which carp were used exclusively, a series of samples was taken from each of the individual fish, which were kept throughout the whole course of each experiment. The fish were sampled by dissecting off narrow transverse strips of muscle from the sides, taking care that each was separated by at least one centimeter from any previously made cut, and that the visceral cavity of the fish was not broken. These samples of muscle were finely minced and two 20-gm. samples weighed out from each. To each of these 50 c.c. of water was added, and they were allowed to stand for at least 30 minutes with frequent shaking.

The proteins were precipitated by adding 20 c.c. of 10-per cent trichloracetic acid to 10 c.c. of muscle suspension further diluted with 10 c.c. of distilled water. These mixtures were allowed to stand for three hours before filtering.

For the tyrosine reaction five c.c. of the tyrosine reagent and 20 c.c. of 15-per cent sodium carbonate were added to from one to 10 c.c. of the trichloracetic acid filtrate, depending on the approximate amount of free tyrosine present, in a 50-c.c. volumetric flask, which was then filled to the mark with distilled water. Color comparison was made after the reaction mixture had stood for one hour, in a Bausch and Lomb colorimeter, with a standard which contained .5 mg. of tyrosine in the same volume and which was set at 10 or 20 mm., depending on the amount of color developed in the unknown.

The formation of cloudy reaction mixtures was far less frequent when 15 per cent of sodium carbonate was used in place of a saturated solution, as directed by Folin and Denis (1912a). The color

developed more slowly in the former case, but at the end of an hour the depth of color in a mixture which contained 20 c.c. of 15-per cent sodium carbonate was the same as in one with the same amount of free tyrosine and 20 c.c. of saturated sodium carbonate. When a reaction mixture did become cloudy it could be cleared by centrifuging without loss of coloring material, the precipitate being white.

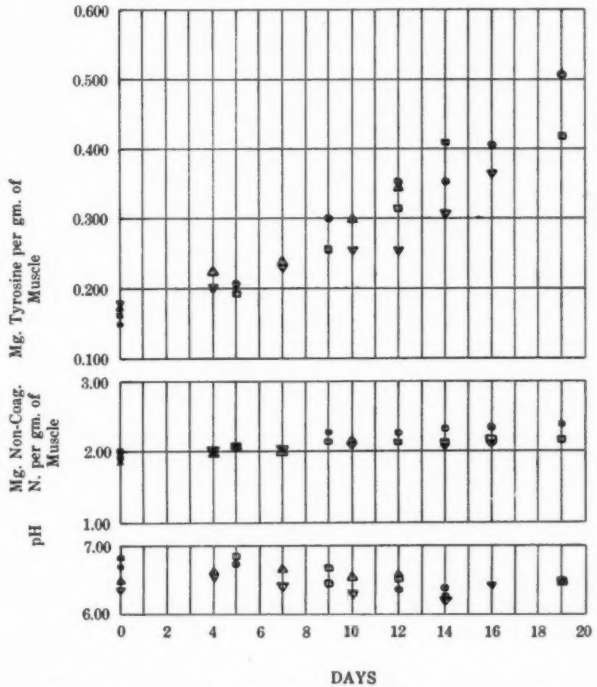

FIG. 1. pH, noncoagulable nitrogen, and tyrosine values of carp stored at .6 to 2.8°C.(33 to 37°F.).

The pH was determined with a Coleman pH electrometer.

The noncoagulable nitrogen was determined by the Kjeldahl method in the trichloracetic acid filtrate. The nitrogen was determined colorimetrically in an aliquot of the distillate by means of Nessler's reagent.

In the preliminary experiment the fish were stored in a cold room which was maintained at .6 to 2.8°C.(33 to 37°F.). They were sampled at intervals and the tyrosine reaction, noncoagulable nitrogen, and pH were determined. The data obtained (Fig. 1) show that the flesh of each of these fish was soft but untainted on the 12th

day of storage. They had developed a tainted odor by the 14th to 16th day, and those of which any remained for sampling on the 19th day were then quite spoiled.

It is well known that neither the noncoagulable nitrogen nor the pH are satisfactory criteria for measuring the spoilage changes in fish muscle. The differences between the changes in these factors and in the intensity of the tyrosine reaction were, however, so marked that the latter appeared to show some promise as a measure of spoilage, particularly since the increases which were found showed a considerable degree of parallelism to the changes in the muscle as judged by organoleptic examination.

TABLE 1

Tyrosine Reaction of Carp Muscle Stored at 17.8 to 20°C.(64 to 68°F.)

Days of storage			
0	1	2	3
.197[1]	.271	.290	.621
.188	.265	.333	.667
.193	.241	.321	.484
.186	.269	.293	.508
.219	.248	.309	.856
.208	.250	.326	.488
.173	.266	.333	.521
.167	.245	.367	.546
Av., .191	.257	.321	.586

[1] Given in milligram of tyrosine per gram of muscle.

The data appeared to warrant further study of the use of tyrosine reaction as a measure of spoilage, so a more extended investigation was undertaken. Several experiments were carried out in each of which a large number of fish were stored. Several were removed from time to time and a sample of muscle taken from each, the unused portion being discarded. The sampling was done by dissecting off a fillet from one side of each fish. The fillets were treated in the same way as the strips of muscle which were taken as samples in the preliminary experiments. The tyrosine reaction only was determined.

Two more storage experiments were carried out with carp. In the first the fish were stored in a room the temperature of which varied from 17.8 to 20°C.(64 to 68°F.). The fish were placed in a double layer in the bottom of wooden boxes and kept moist by occasionally sprinkling them with water and covering the boxes with burlap sacks to reduce evaporation. The tyrosine reaction of the muscle tissue of eight fresh carp of the same lot was determined.

Eight of the stored fish were removed after one day, eight after two days, and eight more after three days' storage; the data for this experiment are given (Table 1).

The flesh of all the fish was soft but without any "off" odor after 24 hours' storage. After 48 hours some taint had developed, and those remaining were quite spoiled after three days.

In the next experiment carp were eviscerated, washed, and packed in melting ice. Eight of these were removed for analysis at the end of each week of storage (Table 2).

TABLE 2

Tyrosine Reaction of Carp Muscle Stored in Melting Ice

Days of storage				
0	7	14	21	28
.145[1]	.163	.191	.265	.433
.128	.156	.229	.289	.421
.149	.152	.195	.256	.390
.122	.152	.202	.279	.425
.157	.166	.220	.301	.366
.138	.158	.234	.266	.436
.162	.149	.210	.258	.369
.117	.153	.200	.263	.378
Av., .140	.156	.210	.272	.402

[1] Given in milligram of tyrosine per gram of muscle.

The flesh of these fish was slightly soft but quite suitable for use as food after 21 days' storage, but it was quite spoiled after 28 days.

All of the above work was done at the University of Wisconsin. Two experiments were also carried out with salt-water fish, at the Pacific Fisheries Experimental Station of the Fisheries Research Board of Canada, at Prince Rupert, B. C.

In the first of these experiments herring were stored in ice in a room which was maintained at 1.7 to 2.2°C.(35 to 36°F.). Eight were removed at each sampling, and the fillets from pairs were minced together so that only four samples were obtained each time; the tyrosine values found are given (Table 3).

These fish showed some evidence of spoilage on the eighth day of storage. The eyes were opaque and the flesh slightly soft, but no perceptible "off" odor appeared until the 10th day, when some taint could be detected. On the 14th day, they were considered unfit for use as food.

Finally the reaction was applied to humpback salmon. The freshly caught fish were stored in the cold room at 35 to 36°F. They were wiped off individually and wrapped in heavy paper.

Four fish were removed at each sampling; the tyrosine values found are given (Table 4).

On the eighth day of storage these fish showed some evidence of spoilage. The flesh was slightly softened but, although the surface of the fish had a spoiled odor, the muscle was not contaminated. On

TABLE 3

Tyrosine Reaction of Herring Muscle Stored at 1.7 to 2.2°C.(35 to 36°F.)

Days of storage						
0	4	6	8	10	12	14
.240[1]	.320	.516	.492	.576	.566	.632
.280	.306	.558	.574	.524	.558	.654
.256	.360	.570	.574	.586	.616	.720
.276	.412	.506	.486	.584	.640	.622
Av., .263	.349	.537	.531	.567	.595	.657

[1] Given in milligram of tyrosine per gram of muscle.

the 10th day the flesh was markedly softer but still untainted; on the 14th day it was spoiled.

DISCUSSION

The value of a test for the spoilage of a food material such as fish depends mainly on two considerations. In the first place, decomposition products should be detectable by the test before spoilage

TABLE 4

Tyrosine Reaction of Pink Salmon Muscle Stored at 1.7 to 2.2°C.(35 to 36°F.)

Days of storage				
0	4	8	10	14
.260[1]	.196	.356	.358	.388
.220	.188	.340	.460	.384
.262	.216	.362	.402	.356
.188	.208	.356	.339	.326
Av., .232	.202	.353	.389	.363

[1] Given in milligram of tyrosine per gram of muscle.

can be recognized by organoleptic examination. Secondly, the values given by different individuals of the same variety, which are at approximately the same stage of decomposition, should always lie within a fairly narrow range. The range of the values obtained after the appearance of decomposition should never overlap the range of the values obtained before.

In the present work increases in the tyrosine reaction were found in all cases before the ordinary evidences of decomposition, such as

softening and tainted odor, became evident. The early increases in the intensity of the tyrosine reaction were more marked in herring and salmon than in carp. Even in the latter they were, however, of sufficient magnitude to show incipient spoilage before it could be detected by the other means employed here.

It would appear probable that for the three common food fishes studied, the test readily admits of standardization and application as an objective criterion of condition. In studies already reported by Tarr and Bailey (1939) it was found that halibut did not lend themselves to the test because of large individual fluctuations in tyrosine value.[1] Its applicability to each species, however, must be determined upon that species. For those whose problem it is to check and control spoilage of the various food fishes caught and marketed, we believe the reaction offers sufficient promise to warrant further study.

SUMMARY

A delicate test for the products of autolysis and bacterial action is applied to commercial fish as a test for spoilage.

In the three species studied intensively—carp, herring, and salmon—the value of the test compared with the usual subjective criteria of appearance, consistency, taste, and smell is clearly demonstrated.

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[1] It is suggested that the fluctuations are incident to varying degrees of fatigue where fish are caught on handlines or trawls (groundlines). For seined fish, where the period of struggling is fairly uniform, the test appears more generally satisfactory.

VITAMIN B_1 AND VITAMIN B_2 (G) CONTENT OF VEGETABLES AS INFLUENCED BY QUICK-FREEZING AND CANNING[1, 2]

C. R. FELLERS AND W. B. ESSELEN, JR.
Nutrition Laboratory, Massachusetts State College, Amherst, Massachusetts
AND
G. A. FITZGERALD
Central Laboratory, General Foods Corporation, Hoboken, New Jersey

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To date very few controlled studies have been reported in the literature relative to the stability of vitamin B_1 and B_2 (G) in vegetables as influenced by quick-freezing and canning. In view of the importance of these two methods of food preservation, this investigation was conducted to compare them as regards their influence on the vitamin B_1 and B_2 (G) content of asparagus, peas, lima beans, and spinach.

REVIEW OF LITERATURE

Funnell (1937) found that while fresh dwarf-bush lima beans contained 100-150 Sherman-Chase units of vitamin B_1 and 100 units of vitamin G per 100 grams, frozen lima beans contained only a trace of vitamin B_1. This same investigator found that peas contained 200 to 300 units of vitamin B_1 and 100 units of vitamin G per 100 grams and that none of these vitamins was lost during freezing. Rose and Phipard (1937) reported that fresh raw peas were a rich source of vitamin B_1 (three Sherman-Chase units per gram) and that there was no loss of this vitamin owing to freezing. However, 26 per cent was lost as a result of cooking for 15 minutes. Lima beans were found to be only half as potent in vitamin B as fresh peas. Mature peas and lima beans contained only one-half of the vitamin B_1 content of the immature vegetables. They found that both fresh raw peas and lima beans contained one Bourquin-Sherman unit of vitamin G per gram and that there was no loss of this vitamin in either cooking or freezing. They also found that there were considerable differences in the vitamin B and G content of peas and lima beans, depending upon the season and geographical location where they were grown. Eddy, Kohman, and Carlsson

[1] Contribution No. 335 of the Massachusetts Agricultural Experiment Station, Amherst, Mass.

[2] Presented before the Fifteenth Annual Meeting of the Society of Plant Physiologists, Richmond, Virginia, December 28-30, 1938. Abstracted in "Quick Frozen Foods" 1, No. 8, 24. 1939.

(1936) found that peas contained 50 rat-growth units of the vitamin B-complex per 100 grams. Hanning (1936) reported that canned strained peas and spinach contained 8.2 and 1.6 International Units of vitamin B_1 per ounce, respectively. Day (1931) found that spinach contained 100 rat-growth units of vitamin G per 100 grams. Baker and Wright (1935) found this same vegetable to contain 70 International Units of vitamin B_1 per 100 grams.

Williams and Spies (1938) have presented values for the vitamin B_1 contents of vegetables in terms of micrograms of thiamin chloride (pure crystalline vitamin B_1) per gram. They give preferred values for green peas, canned peas, and spinach of 1.5, 1, and .5 micrograms of thiamin chloride per gram. These authors consider that 1.0 International Unit of vitamin B_1 is equivalent to approximately 3.3 micrograms of thiamin chloride.

However, the permanent commission on biological standardization of the Health Organization of the League of Nations, Mellanby (1938), adopted the value of three micrograms of crystalline thiamin chloride as the new international standard for vitamin B_1; that is, one unit of vitamin B_1 is equivalent to three micrograms of thiamin.

Attention is called to the excellent publication of Booher and Hartzler (1939) on the vitamin B_1 content of foods in terms of crystalline thiamin. In their experimental work an estimated precision of the results are 15 to 20 per cent.

EXPERIMENTAL PROCEDURE

In this investigation the samples of the vegetables being studied were carefully controlled. At the location where they were grown the vegetables were quick-frozen by the Birds-Eye method and shipped to Amherst packed in dry ice. Upon arrival in Amherst the frozen samples were held at —28.8°C.(—20°F.) and the fresh [3] and canned samples at 2.2°C.(36°F.) until used. Simultaneously, shipments from the same lots of the freshly harvested vegetables were made to Amherst every 10 days (during the assay period). At the same time a third portion of the same vegetables was canned by the usual commercial methods and likewise shipped to Amherst; that is, shipments of raw, frozen, and canned vegetables were shipped at 10-day intervals during the packing season. The same raw material was used for freezing and canning.

A detailed description of the samples and their treatment is presented (Table 1). Vitamin B_1 and vitamin B_2 (G) assays were begun

[3] The vegetables described as "fresh" in this paper are not strictly freshly harvested inasmuch as they were from two to 10 days old when actually fed to the rats. However, in all cases the raw vegetables were crisp and of good quality owing to being held in cold storage.

TABLE 1

Description of Samples of Asparagus, Peas, Lima Beans, and Spinach[1]

	Asparagus	Peas	Lima beans	Spinach
Variety	Martha Washington	Thomas Laxton	Henderson Bush	Virginia Savoy (rust resisting)
Where grown	Bridgeton, N. J.	Corinna, Me.	Bridgeton, N. J.	Bridgeton, N. J.
Blanching time—frozen and canned	2.5 min.	50 sec.	3 min.	1.75 min.
Blanching temperature	100°C.(212°F.)	100°C.(212°F.)	100°C.(212°F.)	100°C.(212°F.)
Process time and temperature, canned vegetables	23 min. at 115.5°C.(240°F.)	35 min. at 115.5°C.(240°F.)	45 min. at 115.5°C.(240°F.)	50 min. at 122.2°C.(250°F.)
Size of can	No. 2	No. 2	No. 2	No. 2.5

[1] All canned vegetables were pressure-cooled immediately after processing.

on all of the samples as soon as they arrived in Amherst. Both the solid portion and the free-run juice of the canned samples were assayed in order to find out what percentage of these vitamins went into solution in the juice.

The method of vitamin B_1 assay was similar to that described by Richardson and Mayfield (1937). Young rats were placed in individual wire cages with raised screen floors. They were given the vitamin B_1-free basal diet of Chase and Sherman (1931) and distilled water. The rats were depleted of their body stores of vitamin B_1 in 15 to 20 days and their weights ranged from 50 to 70 grams. They were then divided into experimental groups of eight and, in addition to the basal diet, were fed their respective supplements of the vegetable being assayed. The supplements were fed three times a week, with the exception of the canned samples which were fed daily. The assays were run for five weeks. Two levels of each sample were fed. A reference curve of response was obtained by feeding graded amounts of the International Standard for vitamin B_1 to similar groups of depleted animals. The growth responses obtained by feeding the test food materials were used to determine the vitamin B_1 content of the food, expressed in International Units, by comparing them with the responses of the rats fed different amounts of the reference standard (Tables 2 and 3).

The vitamin B_2 (G) content of the samples of vegetables was determined by the rat-growth method of Bourquin and Sherman (1931) and the results compared with the Standard Reference Curve for vitamin B_2 in terms of Bourquin-Sherman rat-growth units. The experimental rats were handled in the same manner as were the vitamin B_1 rats except that they were fed the basal vitamin B_2 (G)-free diet of Bourquin and Sherman. In addition two drops of tiki tiki were fed daily as a source of vitamin B_1. All of the samples assayed were fed at two or more levels, and in most cases good checks were obtained between the two levels; results of the vitamin B_2 (G) assays are given (Tables 4 and 5).

The data showing the effect of quick-freezing and canning on the loss of vitamin B_1 and vitamin B_2 (G) in asparagus, peas, lima beans, and spinach are presented (Tables 3 and 5). In calculating the percentage loss owing to canning the total contents of the can were considered, i.e., both the solid portion and the juice.

DISCUSSION OF RESULTS

The losses of vitamin B_1 in peas and spinach were very little owing to quick-freezing, but asparagus and lima beans lost approximately from a quarter to one-half of their vitamin B_1, respectively,

TABLE 2

Results of Vitamin B_1 Assays of Fresh, Frozen, and Canned Asparagus, Peas, Lima Beans, and Spinach

Sample	Number of rats	Level fed daily	Average weight gain in 5 weeks	Vitamin B_1	Micrograms thiamin
			gm.	*I. U. per 100 gm.*	*γ per 100 gm.*
Fresh asparagus	7	1.31	32.0	65	195
Frozen asparagus	7	1.63	31.5	51	153
Canned asparagus (solid portion)	7	3.51	29.7	23	69
Canned asparagus (juice)	7	2.60	31.5	32	96
Fresh peas	8	0.61	30.0	133	399
Frozen peas	8	0.71	36.1	130	390
Canned peas (solid portion)	8	1.67	26.1	45	135
Canned peas (juice)	8	1.74	38.8	59	177
Fresh lima beans	8	1.00	21.6	67	201
Frozen lima beans	8	2.00	17.4	31	93
Canned lima beans (solid portion)	8	6.00	23.0	12	36
Canned lima beans (juice)	8	6.00	18.2	11	33
Fresh spinach	8	2.00	24.3	36	108
Frozen spinach	8	2.30	22.6	34	102
Canned spinach (solid portion)	8	5.00	29.5	20	60
Canned spinach (juice)	8	7.00	23.0	10	30

TABLE 3

Loss of Vitamin B_1 in Asparagus, Peas, Lima Beans, and Spinach Owing to Freezing and Canning[1]

	Asparagus	Peas	Lima beans	Spinach
Fresh, I. U. per 100 grams	65	133	67	36
Frozen, I. U. per 100 grams	51	130	31	34
Loss owing to freezing (per cent)	22	3	54	6
Canned, solid portion, I. U. per 100 grams	23	45	12	20
Canned, liquid portion, I. U. per 100 grams	32	59	11	10
Weight of fresh vegetable per can (grams)	300	377	365	485
I. U. vitamin B_1 from fresh vegetable in can	195	501	245	175
Weight of solid portion in can (grams)	304	389	393	488
I. U. vitamin B_1 in solid portion in can	70	175	47	98
Weight of liquid portion in can (grams)	279	212	200	274
I. U. vitamin B_1 in liquid portion in can	89.3	125	22	27
Total I. U. vitamin B_1 in entire can	159.3	300	69	125
Loss of vitamin B owing to canning (per cent)	18	40	72	29

[1] International Units only are used in this table; 1 I. U. = 3γ thiamin.

TABLE 4

Results of Vitamin B_2 (G) Assays on Fresh, Frozen, and Canned Asparagus, Peas, Lima Beans, and Spinach

Sample	Number of rats	Level fed daily	Average weight gain in 5 weeks	Bourquin-Sherman units vitamin B_2 (G)	Riboflavin
		gm.	*gm.*	*Units per 100 gm.*	*γ per 100 gm.*
Fresh asparagus	8	2.5	18.7	53	160
Frozen asparagus	8	2.0	13.9	50	150
Canned asparagus (solid portion)	7	2.5	11.6	33	100
Canned asparagus (juice)	7	5.0	7.6	20	60
Fresh peas	8	1.7	16.6	70	210
Frozen peas	8	1.6	19.0	73	220
Canned peas (solid portion)	8	3.0	20.6	48	143
Canned peas (juice)	8	3.2	19.2	40	120
Fresh lima beans	8	1.0	17.8	122	365
Frozen lima beans	8	1.1	14.0	100	300
Canned lima beans (solid portion)	8	1.6	14.1	62	187
Canned lima beans (juice)	8	2.5	11.7	34	100
Fresh spinach	8	1.4	32.2	145	436
Frozen spinach	8	1.4	23.1	110	330
Canned spinach (solid portion)	8	2.5	15.1	43	130
Canned spinach (juice)	8	4.2	26.7	40	120

TABLE 5

Loss of Riboflavin (Vitamin G) in Asparagus, Peas, Lima Beans, and Spinach Owing to Freezing and Canning[1]

	Asparagus	Peas	Lima beans	Spinach
Fresh, units vitamin B_2 (G) per 100 grams	53	70	122	145
Frozen, units vitamin B_2 (G) per 100 grams	50	73	100	110
Loss owing to freezing (per cent)	6	0	18	20
Canned, solid portion, units per 100 grams	33	48	62	43
Canned, liquid portion, units per 100 grams	20	40	34	40
Weight of fresh vegetable per can (grams)	300	377	365	485
Units vitamin B_2 (G) from fresh vegetable in can	159	264	445	703
Weight of solid portion in can (grams)	304	389	393	488
Units vitamin B_2 (G) in solid portion of can	100	187	244	210
Weight of liquid portion in can (grams)	279	212	200	274
Units vitamin B_2 (G) in liquid portion of can	56	85	68	110
Total units vitamin B_2 (G) in entire can	156	272	312	320
Loss of vitamin B_2 (G) owing to canning (per cent)	2	0	30	55

[1] Bourquin-Sherman units of vitamin G (B_2) are used in this table.

as a result of the quick-freezing process (Table 3). In spinach it appears unlikely that more B_2 should be lost than B_1 as a result of the freezing and canning processes. The authors feel that the value obtained for the raw spinach may be too high. It is interesting to note that the order of loss of vitamin B_1 in the different vegetables as a result of quick-freezing tends to vary directly with the blanching time (Table 1). In all probability this loss of vitamin B_1 is associated with the solubility of B_1 in the blanch water. It seems reasonable to expect that much of the loss of vitamin B_1 during the quick-freezing process may be traced back to the blanching, the loss of the vitamin varying directly with the length of the blanching period.

There was only a small loss of vitamin B_2 (G) in asparagus, peas, lima beans, and spinach during the quick-freezing process (Table 5). During canning, the losses of vitamin B_1 in spinach, lima beans, and peas were larger than during quick-freezing. In general, losses of vitamin B_1 were considerably greater than losses of vitamin B_2 in both freezing and canning.

Williams and Spies (1938) state that the amino group of the pyrimidine nucleus of vitamin B_1 (thiamin) is said to be capable of reacting readily with aldehydes, and that inasmuch as the amino group is essential to physiological activity, this possibility cannot be ignored as a factor in the thermolability of vitamin B_1. Aldehydes are of very common occurrence in foods. Evidence is rapidly accumulating that thiamin occurs in tissues partly in the free form and partly as the pyrophosphate, and there may exist marked differences of thermolability between them. Thus the stability of vitamin B_1 in vegetables during canning may be tied up with the form in which it exists in the vegetable and the amount of aldehydes in the vegetable.

The evidence presented (Table 5) indicates that there is less loss of vitamin B_2 (G) during quick-freezing than during the canning of spinach and lima beans. As in the case of vitamin B_1 there was considerable variation in the loss of vitamin B_2 (G) during canning in different vegetables. There was no loss in peas, a moderate loss in asparagus and lima beans, and a marked loss in spinach. Neither freezing nor canning markedly injured the vitamin G in asparagus or peas.

SUMMARY

Controlled samples from the same lots of asparagus, peas, lima beans, and spinach were quick-frozen and canned by commercial methods and their vitamin B_1 and vitamin B_2 (G) content was compared with that of the raw vegetables. Fresh asparagus, peas, lima

beans, and spinach were found to contain 65, 133, 67, and 36 International Units of vitamin B_1, and 53, 70, 122, and 145 Bourquin-Sherman units of vitamin B_2 (G) per 100 grams, respectively. Asparagus, peas, lima beans, and spinach retained 78, 97, 46, and 94 per cent of their vitamin B_1 and 95, 100, 84, and 91 per cent of their vitamin B_2 (G), respectively, when quick-frozen; and 72, 60, 28, and 71 per cent of their vitamin B_1, and 98, 100, 70, and 45 per cent of their vitamin B_2 (G) content, respectively, when canned.

The length of the blanching period may influence the vitamin B_1 and B_2 (G) content of quick-frozen and canned vegetables. Because of the solubility of these vitamins in water, minimum blanching periods consistent with the requirements are recommended.

In conclusion, it may be stated that both quick-frozen and canned vegetables retain nutritionally significant percentages of vitamins B_1 and B_2 (G) and both types of preserved foods are good sources of these two water-soluble vitamins.

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ONION JUICE AND BACTERIAL GROWTH[1]

JAMES E. FULLER AND ERNEST R. HIGGINS
Massachusetts Agricultural Experiment Station, Amherst, Massachusetts

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For many years there has been a popular belief that garlic and onions can prevent the growth of bacteria, and in recent years some laboratory studies on the subject have been made. Walker, Lindegren, and Bachmann (1925) studied the toxicity of juice extracted from succulent onion scales for fungi causing onion rot. They found that the fungi could infect sound onion tissue but could not survive in the concentrated extracted juice. Walton, Herbold, and Lindegren (1936) obtained evidence that vapors from crushed garlic contain a strong bactericidal substance. Later, Vollrath, Walton, and Lindegren (1937) and Ingersoll, Vollrath, Scott, and Lindegren (1938) suggested that acrolein, crotonaldehyde, or some closely related compound may be the active bactericidal substance in garlic vapors. Lovell (1937) reported that onion vapors have a bactericidal effect that is weaker and is more quickly lost than that of garlic vapors.

In the study reported in the present paper the interest was in the possible influence of uncooked onion tissue or juice on the growth of bacteria.

EXPERIMENTAL PROCEDURE

Two varieties of onions were employed in the study. One was a Yellow Globe variety grown extensively in the Connecticut Valley. These onions were purchased soon after they had been harvested, and part of the supply was used for immediate study. The remainder was stored in the college cold-storage plant for four months at about 1.7°C.(35°F.) to test the effect of cold storage. The second variety was a white Texas onion purchased at a local market.

Sound, unsprouted onions were selected for the experiments, and the roots and dried outer scales were discarded. An Enterprise grinder was used to reduce the onion tissue to pulp, and the juice was extracted from the pulp with a screw press. The grinder and press were carefully washed and then steamed in an Arnold sterilizer before they were used. Juice was received into sterile containers and aseptic technique was employed for all steps after the juice

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left the press. With the equipment available, however, it was impossible to produce sterile juice, as is shown later in this report.

Lovell's experiment was repeated with the two varieties of onions, and the freshly cut tissue of both was found to possess the property of inhibiting bacterial growth. Experiments with juice expressed from ground onion pulp showed that the growth-inhibiting vapor had been lost during the grinding and extracting process. This result agrees with Lovell's statement that onion tissue loses much of its bactericidal vapor within ten minutes after it is ground.

The first experiment with the onion juice was to study its spontaneous fermentation when exposed to air contamination. Plate counts were made from the freshly extracted juice, the media used being nutrient agar for the bacteria counts and peptone-glucose-acid agar, Fred and Waksman (1928), for mold counts. One series of plates was incubated at 25°C.(77°F.) and one at 37°C.(98.6°F.). Plates were observed at 48 hours, and the mold plates again at seven days. Results showed bacteria counts of 8,800 per ml. of juice at 25°C. and 4,400 at 37°C. At 25°C. the peptone-glucose-acid plates were overrun with molds, and at 37°C. the count was about 2,000 per ml. These counts showed that the freshly extracted juice was not sterile.

One litre of the juice was kept in a sterile flask stoppered with cotton and allowed to ferment at room temperature, about 23°C. (73.4°F.). The decomposition of the juice proceeded as an acid fermentation. The number of bacteria per milliliter increased somewhat up to five weeks and then gradually decreased until, at ten weeks, no bacteria grew on plates made from the juice. No molds could be isolated from the juice after two weeks. The exact organism causing the fermentation was not studied, because the investigation was concerned with possible bactericidal properties of the juice.

Since the juice obtained by the method described above was not sterile, and bacteria were found to multiply in it, the necessity of sterilizing the juice was obvious if any study was to be made of its effect on pure cultures of bacteria. The use of the Seitz filter was chosen as the method of sterilization least likely to denature the juice or interfere with its possible growth-inhibiting properties. Juice so sterilized was tubed aseptically and added to quantities of nutrient broth prepared so that all the tubes contained the same concentration of nutrients (peptone and beef extract) except the tubes containing undiluted onion juice. The juice was used in concentrations of 1, 5, 10, 20, 30, 40, 50, 60, 80, and 100 per cent.

The bacteria employed were strains of *Aerobacter aerogenes*, *Escherichia coli*, *Salmonella schottmuellerei*, *Pseudomonas pyocy-*

anea, Staphylococcus aureus, Proteus vulgaris, and five species of the *Bacillus subtilis* group. These bacteria were chosen to represent both ordinary air contaminants and species from human source. The latter included species that may cause food poisoning. Two or more strains of each species were employed. Inoculations were made from physiological saline suspensions of 24-hour agar-slant cultures of the bacteria. Inoculated tubes were incubated at 37°C.(98.6°F.) and were examined for visible growth at 48 hours and seven days. The quantity of growth was estimated on the basis of turbidity and was compared with that in nutrient-broth cultures with no onion juice. At each examination period a loopful of culture from each tube was streaked on an agar slant to determine the viability of the cultures.

RESULTS OF EXPERIMENTS

The following outline summarizes the results of the experiment. Growth in the medium equal to that in the nutrient-broth cultures is indicated as 4+; decreasing quantities of growth are indicated as 3+, 2+, etc.; and 0 indicates no visible growth. The percentages are concentrations of filtered onion juice.

1. *Aerobacter aerogenes:* 4+ through 100%. All cultures viable after seven days' incubation.

2. *Escherichia coli:* 4+ through 20%; 3+, 30 to 60%; 2+, 80%; —100%. All cultures viable after seven days' incubation.

2. *Escherichia coli:* 4+ through 20%; 3+, 30 to 60%; 2+, 80 to 100%. All cultures viable after seven days' incubation.

3. *Salmonella schottmuellerei:* 4+ through 5%; 3+, 10 to 20%; 2+, 30 to 50%; +, 60%; 0, 80 to 100%. All cultures viable after seven days.

4. *Pseudomonas pyocyanea:* 4+ through 60%; 2+ at 80%; 0, at 100%. All cultures viable after seven days.

5. *Staphylococcus aureus:* 3+, at 1%; 2+, at 5%; +, 10 to 30%; 0, 40 to 100%. All cultures viable after seven days.

6. *Proteus vulgaris:* 4+, through 5%; 2+, at 10%; 0, 20 to 100%. All cultures viable after seven days.

7. *Bacillus subtilis:* 4+, through 5%; 0, 10 to 100%; cultures viable after 48 hours through 40%, and through 10% after seven days. Cultures of *B. cereus, B. megatherium, B. mycoides, B. mesentericus,* and *B. simplex* gave results similar to those obtained with *B. subtilis.* Cultures of the *B. subtilis* group heated to kill vegetative cells gave practically the same results.

These results indicate that the filtered onion juice had some ability to inhibit growth of all of the bacterial strains employed except *Aerobacter aerogenes.* Only members of the *B. subtilis* group

were actually killed. The bacteriostatic agent in the vapor had all been exhausted, and yet some remained in the juice. No attempt was made to determine the nature of this agent because it was not present in the onion juice in sufficient strength to be of any practical value in food preparations containing onion tissue. The findings detailed above were obtained with the Connecticut Valley onions. Experiments with the white Texas onions gave similar results, except that the inhibiting power of their juice was slightly weaker.

Several other points were investigated which are summarized as follows:

1. The bacteriostatic property of the onion juice was substantially weakened by intermittent sterilization in an Arnold sterilizer, and it was practically destroyed by sterilization in the autoclave.

2. Control of pH values, from pH 5.8 (of the freshly expressed juice) by degrees to pH 8, had no effect on the bacteriostatic power of the filtered juice.

3. Storage of onions for four months at 1.7°C.(35°F.) had no demonstrable effect on the bacteriostatic property of the filtered juice.

SUMMARY

Studies were made of the growth-inhibiting property of onion juice for several species of bacteria. The onion varieties employed were Yellow Globe onions grown locally, and a white onion from Texas. Bacterial species employed represented both air-borne contaminants and species that usually come from human source. The freshly expressed juice was not completely sterile. Juice filtered through a Seitz filter had lost all of its bactericidal vapor, but still retained some power to inhibit bacterial growth when it was incorporated in a fluid medium.

Growth of bacteria from human source was not inhibited sufficiently to suggest any practical value of the property. The onion juice was more effective in preventing growth of bacteria of the *B. subtilis* group. Live steam (Arnold) greatly weakened the potency of the juice, and autoclave sterilization practically destroyed it. A pH range of from 5.8 to 8 had no effect on the bacteriostatic property of the juice, and the same was true of cold storage for four months.

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COLOR IN CALIFORNIA WINES

III. METHODS OF REMOVING COLOR FROM THE SKINS

MAYNARD A. AMERINE AND WILLIAM DEMATTEI
Division of Viticulture, University of California, Davis, California

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The anthocyanin pigments responsible for the color of red wines are present in the epidermal cells of the grapes in most varieties of *Vitis vinifera* (L.), although a few varieties, such as Alicante Bouschet, Grand noir, and Salvador, also have a pigmented juice. In the normal procedure for making red wines the color is released from the cells of the skins during fermentation. Whether the release of pigment from the cells is the result of the loss of their semipermeability owing to asphyxiation, to killing by alcohol, to changes in pH favoring release of color, or to death from other causes, such as heat, is not known. Any treatment, however, that kills the cells of the skin results in the loss of the semipermeability of these cells and the consequent release of the anthocyanins.

Little difficulty is experienced in the production of dry red wines in securing the extraction of a maximum amount of pigment from the skins during fermentation, since the period of time during which the grapes are in contact with the skins may extend from three to six days. Also, during the later stages of this contact the skins may be submerged in or have pumped over them a six to 10 per cent alcoholic solution of their fermenting must. In the making of red sweet wines, on the contrary, the period of time before drawing the fermenting must from the skins for fortification is much shorter, frequently only 36 hours, hence the amount of color extracted is sometimes rather limited. This deficiency in pigmentation is a serious difficulty for the sweet-wine industry, since the American consumer demands that ports must have a full red color. Furthermore, red sweet wines are commonly produced in the districts of California (such as Fresno) where the total color content of the grapes is low, Winkler and Amerine (1938a). In these districts the fermentation period may be quite short, too, owing to the higher temperatures during the vintage, unless considerable artificial cooling of the must or other means of reducing the rate of fermentation is resorted to. In addition, the pH of these musts and wines is so high as to reduce the brightness of the color. Finally, the varieties of grapes most widely planted in California for the production of red sweet wines at present are not as rich in color content as they

should be, especially in the warm seasons. Only the Alicante Bouschet of the varieties commonly used — Petite Sirah, Carignane, Mataro, Zinfandel, Trousseau, and Grenache — always furnishes sufficient color in the new wine, but other factors—flavor and instability of color—prejudice most wine makers against its use. The Petite Sirah can be manipulated in all except the very warmest seasons to give sufficient color (by punching down frequently, etc.) and usually the Zinfandel can be handled to yield a properly colored wine. In the cooler districts and in the cool seasons the Mataro and Carignane may produce a fairly normal red sweet wine, but under such conditions they are better suited for making dry red wines than for sweet wines. Grenache, and especially Trousseau, rarely have sufficient color and must be blended with some very dark variety (such as Salvador) to make a suitably colored commercial port wine.

Several previous studies have been made on the extraction of color from the skins of California grapes. Joslyn, Farley, and Reed (1929) studied the effect of temperature and time of heating on the intensity of color and tannin content of red grape juice extracted from *crushed* Petite Sirah grapes. They found very little increase in extraction by changing the temperature from 40 to 50°C.(104 to 122°F.), but a more rapid increase from 50 to 70°C.(104 to 158°F.), and a very large increment by raising the temperature from 70 to 90°C.(158 to 194°F.). Little change was noted by heating at 100°C. (212°F.) compared with 90°C.(194°F.). The tannin content of the heated samples changed in a similar fashion. This confirms in a quantitative fashion previous work by Cruess (1920) who found the color was dissolved from the skins of crushed grapes into the juice in five minutes at 71.1°C.(160°F.).

Bioletti (1905 and 1906) conducted laboratory and plant experiments on the heating of free-run juice from crushed grapes and then placing the heated juice back on the skins. The hot juice rapidly became colored and the whole mass was pressed within a short time. The juice was cooled and fermented in closed containers. The advantages of the method appear to be that there was a partial pasteurization of the must and that cleaner, sounder wines could be made in the closed containers. On the other hand, the danger of overoxidation in pouring hot juice over the aerated, crushed skins should be recognized, as well as the manipulative difficulties.

Dubaquié (1926) found that placing whole grapes in a steam atmosphere for 24 hours increases the color of the wine made from the grapes as well as giving it a special and desirable aroma. Ferré (1926) modified the traditional practice by which crushed grapes

are heated by placing entire grapes in boiling water for one to three minutes in order to kill the cells of the skin and then cooling rapidly to prevent any effect of prolonged heat. He reports that the color rapidly diffuses from the skin into the pulp and that within six to 12 hours most of the color is found in the juice. This fact has been confirmed in numerous tests in this laboratory. Ferré also ran a jet of superheated steam on grapes and obtained a similar effect. He points out that by this method red musts may be obtained by simply crushing and pressing the cold treated grapes and that the must can thus be handled for fermentation as if it were a white juice. The color was more intense and the alcohol content, extract,

TABLE 1

Effect of Respiration on Color Extractable From Skins

Time	CO_2 in jars	O_2 in jars	Color reading of 100-gram sample heated 20 seconds	Increase in color content	Balling
			38°C.(100.4°F.)		
hr.	*pct.*	*pct.*		*pct.*	
0	.....	....	20.0	.....	21.6
17	29.4	1.3	18.0	11.1	20.1
21	41.4	0.1	13.9	44.0	20.4
40	74.8	0.0	13.6	47.0	20.3
86	97.2	0.0	10.7 [1]	87.0 [1]	18.2 [1]
			24°C.(75.2°F.)		
0	.....	....	20.0	.....	21.6
17	15.0	5.2	19.2	4.1	21.2
21	18.7	2.5	19.2	4.1	20.7
40	23.2	5.4	15.7	27.4	21.1
86	48.7	0.0	13.4	49.2	20.0

[1]Some fermentation had taken place in berries having cracked skins.

tannin, and aroma were increased. All of our tests have been made using uncrushed grapes.

TESTS WITH GAS TREATMENTS

Carignane grapes were carefully picked in order to break as few berries as possible. Twenty pounds were placed in wide-mouth, five-gallon glass jars. Each of the jars was filled simultaneously so that there was a random selection of fruit. Four jars were closed and placed at 38°C.(100.4°F.) and four at 24°C.(75.2°F.). At intervals of 17, 21, 40, and 86 hours one jar was removed from each room and the air around the grapes was analyzed for CO_2 and O_2, and the grapes for degree Balling and color content of the juice. A check, similarly selected, was used for determining the original degree Balling and color content (Table 1). To measure the color of the

samples, 100 grams of grapes were selected from each lot, then crushed in a beaker, and heated for 20 seconds, while stirring, on a boiling-water bath. The juice was cooled and filtered as rapidly as possible. The check was fresh grapes of the same lot treated in exactly the same fashion. The color comparisons were made in a Duboseq colorimeter with the check on one side with the plunger set at 20 mm. Therefore, the most highly colored liquid will have the lowest reading. The 20-second treatment was necessary in order to have enough pigment in the juice of the check samples for comparison. The time of heating was the shortest with which we could obtain consistent results. Greater color differences would have been obtained without heating. The oxygen and carbon dioxide readings were taken with a Standard Burrell Gas Analysis apparatus.

The respiration of grapes at 38°C. is thus seen to reduce the oxygen content of the container to about one per cent within 17 hours. There is also 11 per cent more color[1] in the crushed juice at this time than in the check. Another sample taken a few hours later showed over 40 per cent more color than the check and a large increase in percentage of carbon dioxide. The figures for 86 hours are less reliable owing to fermentation activity in the few cracked fruit present.

In the 24°C. experiment the respiration was slower but a 50-per cent increase in color was obtained in 86 hours. This compared with a similar result for only 40 hours at 38°C. Since the grapes in all except the 86-hour sample at 38°C. were in good condition, the increased color may have been due to the cells of the skin being more permeable owing to external conditions or to an intercellular or intracellular respiration which also increased the permeability or to a combination of both.

It is quite probable that in the later stages of storage at 38°C. considerable anaerobic respiration occurred. (Note the absence of oxygen in the air space after 21 hours of storage.) The products of anaerobic respiration (alcohol and CO_2) would no doubt affect the permeability of the cell wall if they accumulated in sufficient amounts in or around the cells. Perold (1927) and others found the anthocyanin to be present in the three or four outer layers of cells of the

[1]Although the Lambert-Beer law is not exactly obeyed, one may use the usual equation to calculate the percentage change in color. If

$$C_x = C_s \frac{l_s}{l_x},$$

where C_s and C_x is the concentration of color in the known and unknown and l_s and l_x the lengths of the plungers of the colorimeter, then the percentage increase in color (assuming C_s to be 1) is $(C_x - 1) \times 100$. This method of calculating the relative color change has been used throughout.

epidermis. Babo and Mach (1933) reported the pigment to be in solution in the cells. Whatever the cause of the release of color, it is obvious that the permeability of the cells is increased when the grapes are confined to a closed jar.

On the basis of the previous tests it was hoped that changing the gas surrounding the fruit would give more immediate results. Accordingly, 20-pound samples of Carignane grapes were placed in five-gallon glass jars and the air was replaced by an atmosphere of the various gases as indicated (Table 2). The grapes were left 51 hours at 38°C. Untreated samples in closed and open check jars were also maintained under the same conditions. At the end of the

TABLE 2

Effect of Various Gas Treatments on Color Extractable From Skins

Treatment	Initial concentration	Color reading using open check as standard	Increase in color content over open check	Color reading (calculated) compared with fresh grapes at 20.0	Increase in color content over fresh grapes
38°C.(100.4°F.)—51 hours					
	pct.		*pct.*		*pct.*
Open check	.....	20.0	.....	17.1	17.0
Closed check	.....	17.0	17.8	14.5	38.0
Oxygen	100	14.8	35.0	12.7	57.4
Carbon dioxide	100	16.6	20.5	14.2	40.8
Nitrogen	100	14.2	40.9	12.1	65.2
Ethylene	1:5,000	21.3	—6.1	18.2	10.0
Room temperature—51 hours					
Hydrogen cyanide	75	45.6	—55.2	—39.0	—48.6

experiment a sample of unbroken berries was removed and the fruit crushed and the color determined as in the previous experiment (Table 2).

More conclusive results might have been obtained had the experiments not been conducted for such a long period. The idea that anaerobic respiration is responsible for a considerable amount of the color release seems strengthened by the results of storage in atmospheres of nitrogen or carbon dioxide (40.9 and 20.5 per cent increase in extractable color, respectively). However, the oxygen treatment also indicates that a favorable release of color (35 per cent) occurs when aerobic respiration occurs, although in this case the effect may have been due to secondary results of excessively vigorous aerobic respiration. The ethylene treatment did not seem to change the color extraction, and indeed retarded it somewhat compared with the open check (—6.1 per cent). Stopping of meta-

TABLE 3

Effect of Various Heat Treatments on Color Content of Musts and Wines

Temperature	Time of treatment	Color reading on must	Color reading on wine 11/15/39	Increase over check	Color reading on wine 12/27/39	Increase over check	Total acid 12/27/39	pH 12/29/39	Buffered pH	Color reading on buffered wine	Increase over check
°C.	*min.*			*pct.*		*pct.*					*pct.*
Check	....	∞	12.2	.....	14.0	.....	.57	3.70	3.92	15.3	.....
70	2	35.0	9.2	32.8	7.0	100.0	.49	4.00	4.12	8.4	81.1
86	½	13.9	7.0	74.4	7.2	94.5	.47	4.00	4.10	8.6	77.8
80	1	10.0	8.7	40.2	8.7	61.0	.47	4.05	4.13	9.3	64.8
86	1	13.6	7.9	54.6	6.9	103.0	.39	4.10	4.15	.....	.....
95	3	7.2	6.5	87.8	7.9	77.2	.48	3.90	4.02	8.1	89.0
95	5	7.1	6.0	103.5	8.0	75.0	.37	4.05	4.18	8.5	80.0
95	10	7.1	5.1	139.5	6.5	115.5	.44	3.85	4.00	7.2	112.5
98	1	7.7	6.0	103.5	7.2	94.5	.45	3.87	.....	.....	.....
98	1	9.7	6.5	87.8	8.0	75.0	.45	4.08	4.15	8.7	76.0
Steam	½	12.1	12.0	1.5	11.8	18.7	.45	4.00	.....	.....	.....
Steam	1	11.3	10.2	19.6	8.0	75.0	.48	4.07	.....	.....	.....

bolic activity would not explain the reduced color content of the cyanide-treated samples which was less than that of fresh grapes. The figures for the percentage increase in color compared with fresh grapes are less satisfactory since they were calculated from the data of Table 1, which are not strictly comparable. They indicate, however, the relative maximum changes in color content which were obtained, amounting to a 65-per cent increase in color content in the case of the nitrogen-treated samples. Although greater increases are secured with oxygen and nitrogen and a slightly greater increase with carbon dioxide than for the closed check, the differences are not great.

TESTS WITH HEATING TREATMENTS

Since the storage experiments did not seem to give a satisfactorily large or rapid increase in the extraction of color, heat treatments of *whole* fruit were tried. A preliminary experiment with steam did not give sufficiently reproducible results, consequently most of the tests were made by dipping the grapes in a wire basket into large volumes of water held at given temperatures. In this way each berry was surrounded with water of the desired temperature. The basket was dipped in cold water immediately after the hot-water treatment; thus there was practically no prolongation of the period of heating. The grapes were then crushed and fermented on the skins, with one per cent by volume of a pure culture of Burgundy yeast in must, to 0.0 degree Balling before pressing. A sample of the juice was filtered after crushing and compared with a standard, No. 3 of Winkler and Amerine (1938b); the results are given (Table 3). Carignane grapes were used in this experiment. It is evident from these experiments that there is an immediate diffusion of color from the skins into the pulp after the treatments. Even the one-half minute treatment with 86°C.(186.8°F.) water or with steam gave a marked increase in color present in the freshly crushed grapes. The changes after fermentation are not so large or so spectacular. There is a marked reduction in total acid in all of the treated samples. The pH is thus higher in the treated samples. As shown previously by Winkler and Amerine (1938a), this decreases the brightness of color of the sample. Samples were therefore filtered and the pH of all the samples was brought to approximately 4.0 with buffers. All the samples were diluted the same amount. The figures for the color, after bringing to more nearly the same pH, really represent the true color change of the wines owing to the treatments. Although the results are somewhat erratic, it is obvious that the relative color value of the samples is not changed by changing the pH in this range.

From the first experiments on heating it seemed that considerable amounts of additional color could be secured in the wine from heat

TABLE 4

Effect of Various Heat Treatments on Color Content of Musts During Fermentation and on Resulting Wines

Treatment	Time	0 hours		12 hours		24 hours		36 hours		48 hours		60 hours		67 hours [1]		December 28, 1939				
		Balling	Color reading	Balling	Color reading	Balling	Color reading	Balling	Color reading	Balling	Color reading	Balling	Color reading	Balling	Color reading	Total acid	pH	Color reading	Average color reading	Increase over check
°C.	min.																			pct.
Check	....	25.9	∝	22.3	36.8	22.1	28.5	20.2	24.9	19.9	17.0	13.1	11.9	8.9	9.9	.54	3.99	10.5	10.25	.....
Check	....	25.8	∝	23.1	32.0	21.0	25.0	19.3	23.2	17.7	16.0	10.8	9.9	6.8	8.0	.56	4.00	10.0		
95	1	25.3	26.2	22.9	9.4	21.9	9.0	20.0	8.4	16.5	7.5	10.5	6.6	5.2	5.9	.53	3.97	9.0	8.5	20.8
95	1	26.0	25.9	23.8	10.7	22.8	10.6	21.2	10.0	18.0	9.9	12.2	9.6	7.2	8.8	.47	3.97	8.0		
95	3	25.1	11.9	22.7	8.6	20.2	7.7	18.1	6.4	14.9	6.1	7.4	5.8	5.3	5.5	.40	4.11	6.3	6.8	50.9
95	3	25.9	12.0	23.2	10.9	21.7	9.3	20.5	8.3	16.5	8.2	8.3	7.9	6.1	7.6	.43	4.05	7.3		
98	1	26.0	13.1	23.1	12.8	21.2	10.0	19.0	8.7	15.9	7.9	10.0	7.5	4.7	7.0	.49	3.97	9.0	8.1	26.6
98	1	25.9	12.7	23.2	11.5	22.2	9.9	21.3	9.1	18.0	8.3	10.5	7.9	5.0	7.5	.45	4.05	7.2		
98	3	25.2	10.6	22.7	10.7	19.9	9.1	19.2	8.5	16.0	8.0	7.9	7.6	5.9	4.9	.43	4.00	6.6	6.65	54.2
98	3	25.2	10.9	22.0	10.6	18.1	9.6	16.5	9.0	12.7	8.1	6.0	7.8	3.9	4.6	.44	4.00	6.7		

[1] All the wines were pressed at this stage.

treatments of Carignane grapes. Furthermore, the color was immediately dissolved into the free-run juice so that even the freshly crushed must had a vigorous red tint while the check was practically devoid of color. It occurred to us then that the difference in treated and untreated samples should be even more spectacular in the early stages of fermentation when normally musts destined for making red sweet wines would have to be removed from the skins. The same

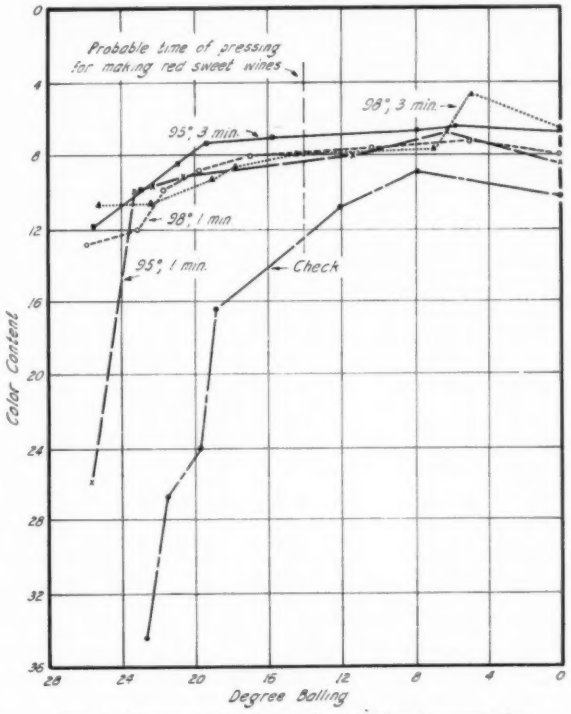

FIG. 1. Changes in color content during fermentation.

experimental procedure was followed as in the previous experiment: 20 pounds of Carignane grapes were treated in hot water at various temperatures. The grapes were immediately cooled and then crushed. Each test was run in duplicate. Samples for Balling and color determinations were removed from the fermentation jars at the beginning and each 12 hours thereafter. The pressing was delayed longer than is commercially practiced but in general the color differences are very well maintained over into the wine; the complete data are given (Table 4).

All of the treated samples showed a very high color content as soon as they were crushed, and after 12 hours' fermentation there was only a small increase in the color content of all the treated musts. The samples which were heated only one minute at 95°C. (203°F.) released somewhat less color at the start but most of the color was extracted within 12 hours. The treatment appears also to have made the color more readily extractable, since the check samples do not show the same rapid increase in color content when the release of color begins. The untreated grapes, however, continued to show a release of color until they were pressed.

By plotting color content against degree Balling (Fig. 1) a better indication of the changes may be obtained. Not until the fermentation is practically finished does the color content of the check approximate that of the treated samples.

TABLE 5

Color Content of Treated and Untreated Samples at 14 and 16 Balling

Treatment	16 Balling		14 Balling	
	Color reading	Increase compared with check	Color reading	Increase compared with check
		pct.		*pct.*
Check	15.2	.....	12.6	.....
95°C.—1 minute	8.7	74.8	8.4	50.0
98°C.—1 minute	8.1	87.8	7.9	59.5
95°C.—3 minutes	7.1	114.0	7.0	80.0
98°C.—3 minutes	8.5	79.0	8.3	51.9

It is of interest to see the difference in color content when the Balling is 14 and 16—the range when the must would be pressed for making red sweet wines. It is indicated that the treated samples contain from about 50 to 114 per cent more color than the untreated check (Table 5).

The experiments indicate that considerably more color may be obtained from treated grapes at the time of normal pressing for making red sweet wines. Even treated samples, which are fermented to dryness on the skins, show some increase in color over the checks. However, whether or not the increase is sufficient to be commercially feasible and whether or not the treatments otherwise adversely affect the quality of the musts is not known. Experiments in the actual production of sweet wines from various varieties of grapes and careful determination of the possible influences on their quality therefore seem necessary. It is stressed that no more color can be obtained from the skins than is normally present, so that even if 100 per cent of the color should be extracted from a variety such as Trousseau, the re-

sulting color of the wine would not be great. It is also of some importance to note that the treated samples show a rapid diffusion of color from the skins into the pulp within an hour or two, so that finally the skins contain practically no color. This seems to show that all color becomes dissoluble in the must after heat treatments such as these. Also, the experiments where the grapes were heated five and 10 minutes showed little, to no, greater color content than samples heated a shorter period, indicating that most of the color must have been released.

SUMMARY

The influence of respiration, various gas treatments, and heat on the rate of extraction of color from uncrushed grapes has been determined. The best results were obtained by dipping the whole grapes in hot water at 95 to 98°C.(203 to 208.4°F.) from one to three minutes. Practically all of the pigments in the skins diffused into the pulp immediately after such treatments. The musts of treated samples contain more color throughout fermentation. At the time of normal pressing for red sweet wines the musts of treated samples contain from 50 to over 100 per cent greater pigmentation than the check samples.

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EFFECT OF LEVEL OF DIETARY RIBOFLAVIN UPON QUANTITY STORED IN EGGS AND RATE OF STORAGE [1]

L. C. NORRIS AND J. C. BAUERNFEIND
Department of Poultry Husbandry, Cornell University, Ithaca, New York

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Kuhn, György, and Wagner-Jauregg (1933a, 1933b, 1933c) found that riboflavin from eggs possesses vitamin B_2 (G) activity. In doing this they used the method of Bourquin and Sherman (1931), modified so as to make the basal diet adequate in a factor necessary to prevent dermatitis in the rat. Previously Aykroyd and Roscoe (1929), in studying the vitamin B_2 (G) content of foods, showed that egg yolk contains the vitamin; and Chick and Roscoe (1929) showed that egg white contains it. Roscoe (1931) reported that fresh egg yolk is richer in vitamin B_2 (G) than fresh egg white but that upon drying egg white is the richer.

Using the method of Bourquin and Sherman (1931), Todhunter (1932-33) found that fresh whole egg contained 1.3 units of vitamin G per gram and Morgan and Hunt (1935), using a method similar to that of Bourquin and Sherman (1931), found that fresh whole egg contained 1.2 units of vitamin G per gram. Upon converting these values into riboflavin by means of the factor of Ellis and Zmachinsky (1937), they become respectively 3.8 and 3.5 micrograms of riboflavin per gram. Snell and Strong (1939), using a microbiological method of assay, reported that fresh egg white contained 3.1 micrograms of riboflavin per gram and fresh egg yolk 7.6 micrograms per gram. According to these values fresh whole egg would contain approximately 4.6 micrograms of riboflavin per gram.

The determination of the riboflavin content of eggs by chemical procedures was attempted by von Euler, Adler, and Schlötzer (1934) who reported that fresh egg yolk contained five to six micrograms of riboflavin per gram and fresh egg white four to five micrograms per gram. According to these values fresh whole egg would contain approximately 4.3 to 5.3 micrograms of riboflavin per gram. Lepkovsky, Taylor, Jukes, and Almquist (1938) also determined the riboflavin content of fresh egg white chemically and reported an average value of 2.9 micrograms of riboflavin per milliliter from the

[1] A preliminary report of a part of the results presented in this paper was made at the 31st Annual Meeting of the Poultry Science Association at Cleveland, Ohio, Aug. 1-3, 1939 (Poultry Science, 1939, 18, 400).

eggs of hens receiving no extra riboflavin in the diet and 4.7 micrograms per milliliter of egg white from the eggs of hens receiving extra riboflavin. They found that the biological value of the fresh egg white corresponded to about 60 per cent of the chemical value. This would make the average riboflavin content of the fresh egg white from the eggs of hens receiving no extra riboflavin 1.7 micrograms per milliliter, or about 15 micrograms per gram of dried egg white, and that of the fresh egg white from the eggs of hens receiving extra riboflavin 2.8 micrograms per milliliter, or about 23 micrograms per gram of dried egg white.

Ellis, Miller, Titus, and Byerly (1933) observed a relationship between the vitamin G content of the diet of hens and that of the eggs produced by them but the differences in results were not marked. Heiman (1935) obtained evidence that the vitamin G content of eggs and the intensity of the yellowish-green pigment of egg white is directly related to the vitamin G content of the diet fed the hens. He confirmed the results of Roscoe (1931), showing that dried egg white is richer in vitamin G than dried egg yolk. Bethke, Record, and Wilder (1936) found that, in assays conducted with both rats and chicks, the inclusion of feedstuffs rich in vitamin G in the diet of hens results in the production of eggs with increased vitamin G content. These investigators also found that dried egg white is approximately 2.5 times as rich in vitamin G as dried egg yolk but that on a fresh basis egg yolk is richer. They observed that invariably the egg white from the eggs of hens fed a basal diet supplemented with vitamin G rich feeds possesses a distinctly more yellowish-green color than the egg white from the eggs of hens fed the unsupplemented basal diet.

From the results of the experimental work just discussed, it is evident that eggs contain vitamin G and that the effects ascribed to this vitamin are due chiefly to riboflavin. It is also evident that the vitamin G or riboflavin content of eggs is influenced by the vitamin G content of the diet fed the hens. In an effort to develop this work further, studies were undertaken to determine the extent to which the riboflavin content of eggs is influenced by increasing the riboflavin content of the diet of hens and also to determine the rate of increase and subsequent decrease in riboflavin content. The results of these studies are presented in this report.

EXPERIMENTAL PROCEDURE AND RESULTS

White Leghorn hens, in their first year of egg production, were used in these studies. They were fed a diet which possessed the following composition:

	Per cent
Yellow corn meal	65.5
Wheat bran	20.0
Purified or Argentine casein	8.0
Pulverized limestone	3.5
Steamed bone meal	2.0
Iodized salt	0.5
Cod liver oil, 250 D.	0.5
Manganous carbonate	Trace

According to the values reported by Norris, Wilgus, Ringrose, Heiman, and Heuser (1936), this diet contained approximately 100 micrograms of riboflavin per 100 grams. The riboflavin analyses of the liver preparations and synthetic riboflavin solutions, used to increase the riboflavin content of the basal diet, were conducted according to the procedures of Sullivan and Norris (1939) and Hodson and Norris (1939).

Eggs were collected for analysis during the last two days of each week of the experiments. These were boiled for 15 minutes and then rapidly chilled in cold water. The shells were removed and the egg whites separated from the yolks. In order to hasten drying both the egg whites and the yolks were passed through a 6-8 mesh screen. The screened materials were placed in a wind tunnel and dried at a temperature of 50 to 55°C.(122 to 131°F.). The time required for drying was approximately 16 to 18 hours. After drying the yolks were continuously extracted with carbon tetrachloride for four to five hours to remove the fat. This solvent was found not to remove any riboflavin. Finally the dried egg white and the dried fat-free yolk were finely ground in a small burr mill. A five-gram sample of each batch of egg white and yolk was used in extracting the riboflavin. The procedure followed, in general, hereafter was that of Hodson and Norris (1939). Acid-acetone was the solvent used for extracting riboflavin rather than 0.25N H_2SO_4. The riboflavin values obtained for the dried materials were converted to the fresh basis by applying the results of moisture determinations of representative eggs. The factors for egg white and egg yolk were found to be .133 and .509 respectively.

A study was conducted to determine whether or not the method of Hodson and Norris (1939) would give results on egg white and yolk comparable to those obtained with the microbiological method of Snell and Strong (1939); results of this study are presented (Table 1). Good agreement was obtained between the riboflavin values obtained for egg white and yolk analyzed by these methods. Agreement was also obtained between riboflavin values for egg white

obtained by the Hodson and Norris method and chick bioassays (Table 1). No chick bioassays were conducted on egg yolk.

In order to become familiar with the rate of response and the range of response of the eggs of hens to low and high levels of riboflavin intake, a preliminary experiment was conducted with two lots of 12 hens each. A diet, similar to the basal diet previously described and containing about 125 micrograms of riboflavin per gram, served as the low-riboflavin diet, and a diet formulated from this by substituting 10 per cent of liver meal for casein served as the high-riboflavin diet. The liver meal contained approximately 70

TABLE 1

Results of Determination of Riboflavin in Egg White, Egg Yolk, and Fat-Free Egg Yolk by Chemical and Biological Assays

	Micrograms and riboflavin per gram	
	Chemical	Biological
Chemical [1] and Bacteriological [2] Assays		
Dried egg white 1	20.8	18.9
Dried egg white 2	16.4	16.5
Dried egg yolk 1	5.3	7.4
Dried egg yolk 2	7.2	7.9
Dried egg yolk 1 (fat-free)	13.4	14.0
Dried egg yolk 2 (fat-free)	18.3	19.2
Chemical [1] and Chick [3] Assays		
Dried egg white 3	14.6	16.0
Dried egg white 4	10.2	11.4

[1] Hodson and Norris (1939). [2] Snell and Strong (1939). [3] Modification of the procedure of Wilgus, Norris, and Heuser (1935).

micrograms of riboflavin per gram, and hence the high-riboflavin diet contained about 825 micrograms per 100 grams.

Within two weeks after changing from a normal diet to the low-riboflavin diet the riboflavin content of the fresh eggs decreased from 2.26 micrograms to 1.39 micrograms per gram. The riboflavin content of these eggs was maintained at approximately this level as long as the low-riboflavin diet was fed. The riboflavin content of the fresh eggs of the high-riboflavin lot, on the other hand, increased from 2.26 micrograms of riboflavin per gram to 3.32 micrograms per gram within four weeks after starting the feeding of the high-riboflavin diet, after which only a slight additional increase was made. The riboflavin content of the fresh eggs of both lots returned to the starting level within four weeks after again feeding the hens the normal diet.

In the second experiment three lots of 20 to 23 hens each, in their first year of egg production, were used. The experiment was conducted for four consecutive periods of 28 days each. Riboflavin was added to the basal diet, previously described, by means of liver extract containing 800 micrograms of riboflavin per gram. One lot was fed the basal diet only. The second lot was fed the basal diet supplemented with 240, 480, 720, and 960 micrograms of riboflavin, respectively, for the first, second, third, and fourth experimental periods. The third lot was fed the basal diet supplemented with 720 micrograms of riboflavin per 100 grams for the first and third periods

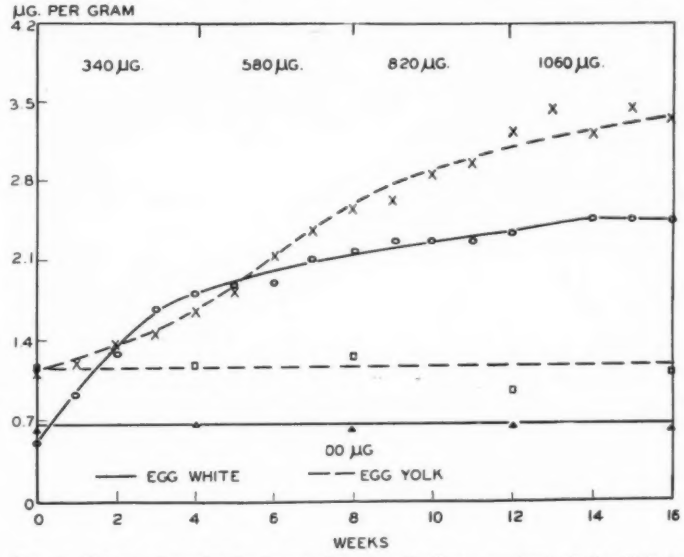

FIG. 1. Effect of gradually increasing the riboflavin content of the diet from 100 to 1,060 micrograms per 100 grams upon the quantity stored in fresh egg white and yolk and the rate of storage and depletion.

and the basal diet only for the second and fourth periods. Throughout the experiment the average weight of the hens in all lots increased slightly, food consumption was adequate, and egg production was satisfactory and consistent.

The riboflavin content of the eggs from the hens fed the basal diet remained fairly constant for the entire experiment. The range in values for the egg white was .64 to .69 microgram per gram, and that for the yolk was .98 to 1.27 micrograms per gram. The weekly riboflavin values for the eggs of the second lot, fed a gradually increasing amount of riboflavin, are presented graphically (Fig. 1).

These values are compared with those for the eggs of the basal lot which, in view of their constancy, are plotted as a straight line. The weekly riboflavin values of the third lot, fed riboflavin every other four-week period, are presented (Fig. 2). The values are also compared with the values of the basal lot, plotted as a straight line.

The gradual increase in the riboflavin content of the diet to 960 micrograms per 100 grams during the experimental period of 16 weeks caused the riboflavin content of the fresh egg white to increase gradually from the average basal level of .59 microgram per gram to 2.45 and that of the fresh yolk to increase gradually

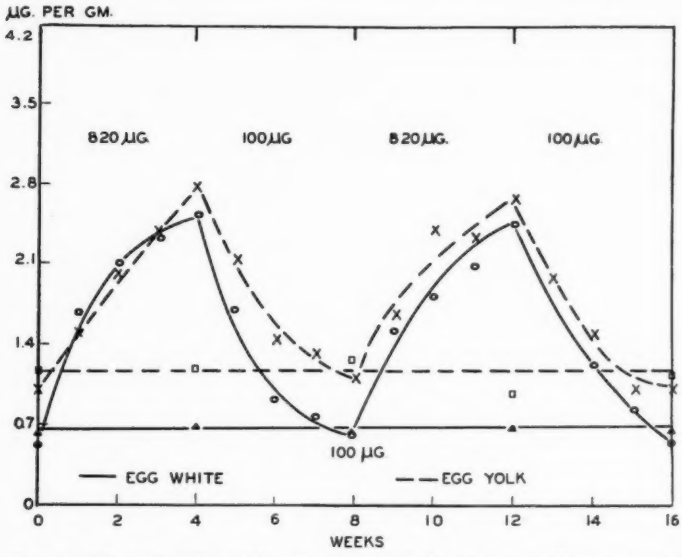

FIG. 2. Effect of feeding 820 micrograms of riboflavin per 100 grams of diet by alternate four-week periods upon the quantity stored in fresh egg white and yolk and the rate of storage and depletion.

from the average basal level of 1.07 micrograms per gram to 3.38. The rate of increase in the riboflavin content of the egg white was greater at the start than that of the yolk but later the rate of increase in riboflavin content was greater in the yolk.

Upon the addition of 720 micrograms of riboflavin to each 100 grams of the basal diet, the riboflavin content of the fresh egg white from the eggs of the hens in the third lot increased from the basal level to 2.55 micrograms per gram and that of the fresh yolk to 2.79 micrograms per gram. The time required to reach these levels was four weeks. Upon discontinuing the feeding of riboflavin the time

required to return to the basal level was also found to be four weeks. These observations were confirmed upon repeating this work with the same hens.

The results of the second experiment gave some evidence of the rate of storage of riboflavin in egg white and yolk but failed to provide convincing evidence that the maximum riboflavin storage levels were attained. The tendency of the riboflavin curves for the egg white and yolk of the eggs from the hens of the second lot in this experiment to plateau, however, is an indication that the maximum storage levels were being approached. In the third and final experiment an attempt was made, therefore, to determine definitely the

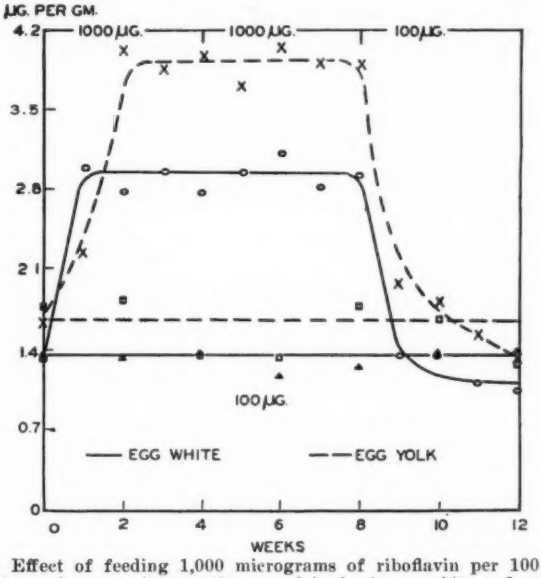

FIG. 3. Effect of feeding 1,000 micrograms of riboflavin per 100 grams of diet for eight weeks upon the quantity stored in fresh egg white and yolk and the rate of storage and depletion.

maximum storage levels of riboflavin in egg white and yolk as well as to obtain further evidence on the rate of storage.

Three lots of 10 hens each, in their first year of egg production, were used in this experiment. The basal diet was the same as that previously described. The experiment was conducted for 12 weeks. One lot was fed the basal diet only, the second lot was fed the basal diet supplemented with 900 micrograms of riboflavin per 100 grams, and the third lot was fed the basal diet supplemented with 1,900 micrograms of riboflavin per 100 grams. Synthetic riboflavin (Merck)

was used to increase the riboflavin content of the diets fed the second and third lots. The riboflavin was mixed into these diets weekly in the form of an alcohol-water solution containing 250 micrograms of riboflavin per milliliter. After mixing, the alcohol was evaporated by exposure to air.

The results of the third experiment (Figs. 3 and 4) show that the riboflavin content of the egg white and yolk from the eggs of the basal lot was maintained again at fairly constant levels. The riboflavin content of the egg white varied from 1.18 to 1.44 micro-

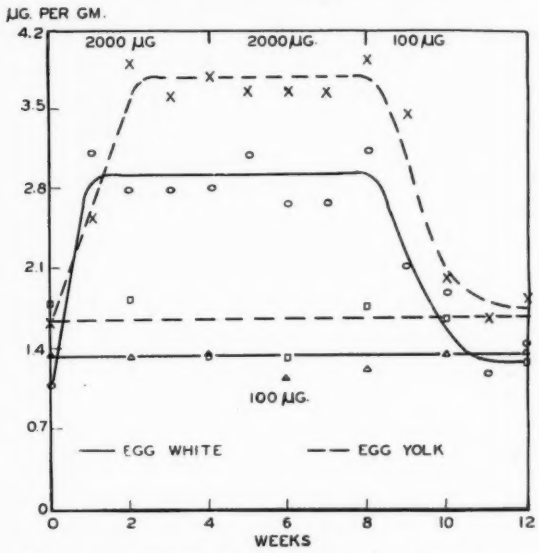

FIG. 4. Effect of feeding 2,000 micrograms of riboflavin per 100 grams of diet for eight weeks upon the quantity stored in fresh egg white and yolk and the rate of storage and depletion.

grams per gram and that of the yolk from 1.33 to 1.78 micrograms per gram. By adding 900 micrograms of synthetic riboflavin to each 100 grams of diet, the riboflavin content of the egg white was increased from the average basal level of 1.29 micrograms per gram to 2.89 and that of the yolk from the average basal level of 1.58 micrograms per gram to 3.98. The addition of 1,900 micrograms of synthetic riboflavin to each 100 grams of diet caused no further increase in the riboflavin content of the egg white or the yolk.

The time required to attain maximum storage of riboflavin in the egg white in the third experiment was approximately one week and that required for the yolk approximately two weeks. After

returning the hens to the basal diet, the time required for the riboflavin content of the egg white from the eggs of the hens which had received 900 micrograms of additional riboflavin per 100 grams of diet to return to the basal level was about one week and that required for the yolk about three weeks. The riboflavin level of the egg white from the eggs of the hens which had received 1,900 micrograms of additional riboflavin per 100 grams of diet returned to the basal level in about three weeks and that of the yolk in about the same period of time. The slower depletion of the egg white from the eggs of the hens of this lot may be an indication of the building up of temporary reserves of riboflavin in the body.

In all experiments the increase in the riboflavin content of the fresh eggs above the basal level was approximately the same (Table 2). It is evident, therefore, that the differences in the maximum ribo-

TABLE 2

Maximum and Minimum Levels of Riboflavin Obtained Experimentally in Fresh Eggs

	Experiment		
	1	2	3
	ug./gm.	*ug./gm.*	*ug./gm.*
Basal diet + riboflavin	3.32	2.77	3.26
Basal diet	1.39	0.82	1.41
Increase in riboflavin content	1.93	1.95	1.85

flavin content of the eggs of the groups of hens in these experiments are due to the differences in the riboflavin content of the eggs when the hens were fed the basal diet only. A possible explanation of this is that the hens used in the first and the third experiments possessed an inherent ability to metabolize riboflavin more efficiently at low dietary levels than the hens used in the second experiment. There appears definitely to be a level above which additional riboflavin in the diet of hens has no effect upon the riboflavin content of the eggs. From the results of these experiments this level appears to lie in the range of 800 to 1,000 micrograms of riboflavin per 100 grams of diet. These values include the amount of riboflavin calculated to be present in the basal diet.

The maximum riboflavin values for the egg white and the yolk obtained in these experiments were compared with those of the egg white and the yolk of eggs produced on poultry farms. Eggs were obtained from hens fed egg mashes and those fed breeder mashes. The latter type of mash generally contains more riboflavin in order to insure normal hatchability, since it has been shown by Norris and

associates (1936) and by others that more riboflavin is required for hatchability than is required for egg production. Eleven flocks of hens are represented in this study of which six were fed egg mashes and five breeder mashes (Table 3).

The average riboflavin content of the fresh egg white from the eggs of the hens fed egg mashes was 1.90 micrograms per gram and that of the yolk 2.39 micrograms per gram. The average riboflavin content of the egg white from the eggs of the hens fed breeder mashes was 2.29 micrograms per gram and that of the yolk 2.85 micrograms per gram. The differences between the values obtained for the egg white and the yolk from the eggs of the hens fed egg mashes and those of the hens fed breeder mashes were statistically significant.

TABLE 3

Riboflavin Content of Farm Eggs

	Riboflavin content
	ug./gm.
AVERAGE OF SIX FLOCKS FED VARIOUS EGG MASHES	
Fresh egg white	1.90 ± 0.12
Fresh egg yolk	2.39 ± 0.07
Fresh egg	2.07 [1]
AVERAGE OF FIVE FLOCKS FED VARIOUS BREEDER MASHES	
Fresh egg white	2.29 ± 0.10
Fresh egg yolk	2.85 ± 0.16
Fresh egg	2.48 [1]

[1] Calculated from assays of egg white and yolk and composition studies of fresh eggs.

In these experiments the highest average riboflavin value obtained for fresh egg white was 26 per cent greater and that of the yolk 40 per cent greater than the average value found in farm eggs. Whether or not it would be economical in practice to secure this increased riboflavin content was not determined in these experiments.

The average riboflavin content of the fresh eggs produced by the hens fed egg mashes was found to be approximately 2.07 micrograms per gram of edible substance (105 micrograms per egg) and that of the eggs from the hens fed breeder mashes 2.48 micrograms per gram (125 micrograms per egg). The lowest riboflavin value obtained experimentally was approximately .82 microgram per gram of edible substance (40 micrograms per egg) and the highest value 3.26 micrograms per gram (165 micrograms per egg). These values are based upon a standard egg weighing two ounces. They are lower than the values reported by Todhunter (1932-33), von Euler, Adler,

and Schlötzer (1934), Morgan and Hunt (1935), and Snell and Strong (1939). The increase in the total riboflavin content of fresh eggs obtained in these experiments was approximately 32 per cent greater than the highest average value found for farm eggs.

SUMMARY

Studies have been conducted to determine the extent to which the riboflavin content of eggs can be increased by increasing the riboflavin content of the diet fed the hens and also the rate of increase in riboflavin content. In these studies it was found that the riboflavin content of fresh eggs could be increased approximately 1.9 micrograms per gram above that of the eggs laid by hens fed the basal diet. The riboflavin content of the latter eggs in one experiment was .82 microgram per gram and in the other two approximately 1.40 micrograms per gram. The time required to attain maximum riboflavin storage in eggs and that required to deplete to the basal levels varied from about two to four weeks. In general, the riboflavin content of egg white changed more rapidly than that of egg yolk. The differences in the basal riboflavin level of the eggs from the groups of hens used in these studies and the differences in the time required to attain maximum riboflavin storage and to return to the basal levels are believed to be due to inherent differences in the ability of the various groups of hens used in these studies to metabolize riboflavin. The highest average riboflavin level obtained in eggs experimentally was about 32 per cent greater than that found in farm eggs laid by hens fed diets designed to promote good hatchability.

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CHEMICAL AND BACTERIOLOGICAL CHANGES IN DILL-PICKLE BRINES DURING FERMENTATION[1]

I. D. JONES, M. K. VELDHUIS, J. L. ETCHELLS, AND OTTO VEERHOFF
Horticulture Department, North Carolina Agricultural Experiment Station, Raleigh, North Carolina

Bureau of Agricultural Chemistry and Engineering, U. S. Department of Agriculture, Raleigh, North Carolina

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The manufacture and storage of genuine dill pickles under climatic conditions typical of the southern states is generally unsatisfactory, and few southern pickle packers attempt to manufacture genuine or fermented dills.

In other sections of the United States several investigators have studied different phases of dill-pickle manufacture. Lesley and Cruess (1928) studied the relationship between brine acidity and soft pickles. Joslyn (1929), in extensive experiments, investigated the influence of numerous commercial practices on the quality of the finished product. Fabian and Wickerham (1935) have reported some chemical and bacteriological changes which take place in dill manufacture.

During the past few years a study of the manufacture of genuine dills under climatic conditions of eastern North Carolina has been undertaken. This paper is a report of a chemical and bacteriological study in regard to the types of microörganisms predominating, the utilization of sugar, and the production of acid during the fermentation. The data presented were obtained during the 1938 curing season.

EXPERIMENTAL PROCEDURE

In outlining the program followed in 1938 one treatment was considered standard; other treatments deviated somewhat from the standard with respect to brine salinity, added acid, or addition of sugar.

The cucumbers used were uniform in size and carefully selected. They were of 600-count size,[2] which would ordinarily be considered too large for marketable dills, but were entirely suitable for making comparisons of different treatments. Duplicate barrels of each treatment were put down.

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[2] Number per 45-gallon cask.

Brine samples were taken at frequent intervals for chemical and bacteriological analyses. These samples were taken by inserting a short piece of stainless steel tubing through the bung of the barrel and withdrawing the brine through an attached piece of rubber tubing. Two 12-ounce bottles full were withdrawn before the bacteriological sample was taken.

Chemical samples were preserved with sodium 2, 4, 5 trichlorphenolate in a concentration of 1-10,000 as described by Veldhuis (1938). The titratable acidity of the brine samples was determined by diluting a 10-c.c. sample with 50 c.c. of water, boiling about 20 seconds to expel carbon dioxide, cooling, and titrating with one-tenth normal sodium hydroxide using phenolphthalein as the indicator. The pH of the brine was determined by means of a glass electrode. Reducing sugars were estimated by the Shaffer-Hartmann (1921) micro method, standardized to the conditions under which it was used. Chemical studies were made on all lots.

For bacteriological analysis, the brine was examined by the plating technic with respect to total number of bacteria, acid-forming bacteria, peptonizing bacteria, and yeasts. Bacteriological findings showed the acid-formers and yeasts to be the predominating organisms and they alone are considered in this study.

The media used for plating of the brine samples were nutritive caseinate agar (Difco) for the bacterial counts and tartaric acid agar for yeast counts. The routine platings of the brine samples on the nutritive caseinate agar were counted and classified according to physiological growth reactions of the predominating organisms. On this medium the acid-formers showed definite zones of precipitated casein about the colony, owing to their acid production. The addition of eight c.c. of .4-per cent brom-cresol-purple indicator to the agar during preparation was a further aid in identification of the acid-forming bacteria. It was found that with the routine use of this media for brine platings, during the active phase of the dill fermentation substantially all of the colonies present on the plates were acid-formers. The yeast counts were obtained by plating dilutions of the brine on tartaric acid agar, which was prepared by adding five c.c. of sterile five-per cent tartaric acid to each 100 c.c. of melted dextrose agar.[3] All plates were incubated at 35°C.(95°F.) for three days and then counted. Occasionally, when yeast colonies were not well developed the incubation period was extended to five days. The treatments followed bacteriologically are outlined (Table 1, Nos. 2, 3, 5, and 11).

[3] Laboratory Manual *(Methods of Analysis of Milk and Its Products)*, International Association of Milk Dealers, 1933. p. 81.

TABLE 1

Salting Schedule Followed in Experimental Dill Pack

(Treatment for all lots was the same as for the standard[1] except as otherwise indicated in this table)

Lot No.	Treatment description	Quantities of materials added per barrel		
		Salt	Acid	Sugar
		lbs.		
1	Standard	19	.5 gal. 110-grain vinegar	None
	Acid series			
	Vinegar—at starting			
2	No acid added	19	None	None
3	Medium acidity	19	1.0 gal. 110-grain vinegar	None
4	High acidity	19	1.5 gal. 110-grain vinegar	None
	Lactic acid— (vinegar omitted)	19		
5	At starting	19	1700 c.c. 50%	None
6	10 days after starting	19	1700 c.c. 50%	None
	Salt series			
7	Low salt	15	.5 gal. 110-grain vinegar	None
8	High salt	23	.5 gal. 110-grain vinegar	None
	Sugar series			
9	Dextrose at starting	19	.5 gal. 110-grain vinegar	3.5 lbs.
10	Dextrose 7 days after starting	19	.5 gal. 110-grain vinegar	3.5 lbs.
11	Sucrose at starting	19	.5 gal. 110-grain vinegar	3.5 lbs.

[1] Standard treatment—19 lbs. NaCl, .5 gal. 110-grain vinegar; barrel filled with water, stored inside shelter, rolled at frequent intervals early. All barrels were of 42-gallon capacity.

DATA AND DISCUSSION

Typical curves of chemical changes which occurred during fermentation in a lot receiving the standard treatment are shown (Fig. 1). Progressive changes in acidity and pH and sugar concentrations of the brine are indicated. From the acidity curve it is evident that the changes in this constituent were small during the first three days. The acid content of .4 gram per 100 c.c. at the end of the first day was due to the vinegar added. During the second and third days the brine acidity decreased somewhat as a result of brine dilution and vinegar absorption by the cucumbers.

A rapid increase in acid content began on the fourth and continued until about the 18th day, followed by a slight decrease, after which there were but small fluctuations in brine acidity.

The sugar curve (Fig. 1) indicates a rapid increase in brine-sugar concentration during the first three days and a correspondingly rapid reduction in this sugar concentration after an active fermentation had started, as indicated by the development of brine acidity.

The pH curve shows the effect of the added acid and acid produced by fermentation. The variations found with this treatment

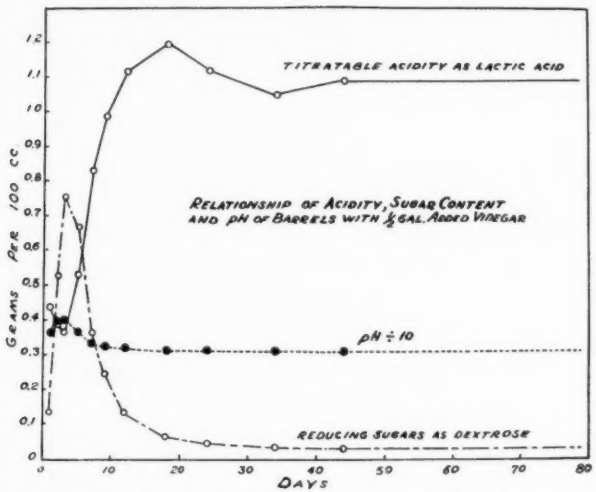
TITRATABLE ACIDITY AS LACTIC ACID
RELATIONSHIP OF ACIDITY, SUGAR CONTENT AND pH OF BARRELS WITH ½ GAL. ADDED VINEGAR
pH ÷ 10
REDUCING SUGARS AS DEXTROSE
GRAMS PER 100 CC.
DAYS

FIG. 1. Chemical changes in the brine of a standard dill treatment during fermentation.

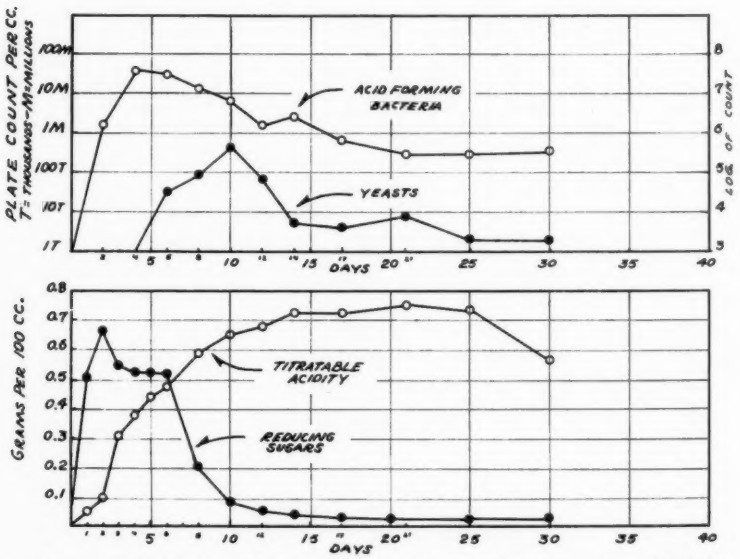

FIG. 2. Relationship between predominating microörganisms in a dill brine and changes in brine constituents. Upper part, growth curves of acid-forming bacteria and yeasts; lower part, sugar utilization and acid formation.

are not very large. The added vinegar lowered the pH to about 3.6 on the first day. During the second and third day the pH rose slightly as the vinegar in the brine was diluted. When the formation of acid by fermentation began, the pH decreased again and continued to decrease slightly as long as acid was formed. A constant value was approached on about the 18th day.

A more or less average dill fermentation is shown (Fig. 2) with respect to predominating microörganisms and their effect upon the sugar present and the production of brine acidity. The upper part shows the differential plate counts of the brine in regard to the acid-forming bacteria and yeasts. Owing to the great difference in the number of the two types, the counts are plotted logarithmically so that both may be shown on the same graph. The acid-forming bacteria show a growth curve of moderate activity, reaching a peak of 38 millions per c.c. on the third day and then subsiding. The yeast fermentation did not begin actively until the acid-forming bacteria had changed the acidity of the brine to more optimal conditions. The yeast population, after starting on the fourth day, reached a peak on the 10th day and then dropped sharply although remaining in the brine for approximately 30 days.

The lower part of Fig. 2 shows the reducing sugar content and titratable acidity of the brine during the fermentation. The acid-forming bacteria brought about the first part of the downward trend of the sugar curve, then the advent of the yeast fermentation aided in bringing about the further decline to approximately .1 per cent on the 10th day. The acidity curve, starting on the first day, shows a gradual rise to approximately .75 per cent on the 21st day, this period comparing favorably with that of the active growth phase of the acid-forming bacteria.

The influence of the addition of lactic acid and different quantities of 110-grain vinegar at the outset of the experiment on subsequent changes in acidity, pH, and sugar concentration of the brine during the fermentation is evident (Figs. 3 to 5). The details of these treatments are shown (Table 1, Treatments 1 to 5).

The quantity of lactic acid added for treatment No. 5 was equivalent to approximately one and three-fourths gallons of 110-grain vinegar. Since the dillweed used in these treatments had been packed in vinegar, a small amount of acid was introduced into all barrels including those designated as "no acid added." The amount of vinegar thus added was equal to about one pint of 110-grain vinegar.

For the vinegar-treatment series the curves (Fig. 3) show that there was a decrease in the acid content of the brines during the first few days as the vinegar became diluted. This condition was

followed by a rapid rise in the acidity as acid was produced by the fermentations. The addition of successively larger amounts of vinegar at the outset had the effect of delaying the start of the acid fermentation for longer periods of time, but did not otherwise materially affect the formation of acid by fermentation. A half gallon of vinegar diluted to 42 gallons results in a solution having an acid content equivalent to about .2 gram of lactic acid per 100 c.c. The maximum acid content attained in lots with one-half gallon of added vinegar was approximately .2 gram per 100 c.c. more than that attained in lots receiving no vinegar; thus the increase was about equal to the added vinegar. In the cases where larger amounts of vinegar were added, the increase in the maximum acidities attained was roughly equal to the added vinegar.

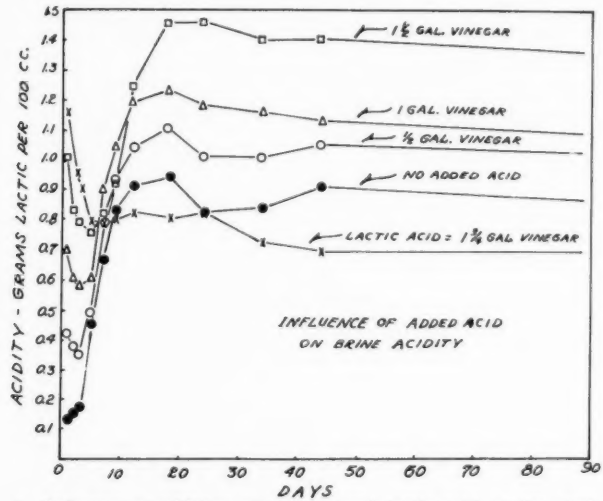

FIG. 3. Influence of addition of vinegar and lactic acid at the start of dill fermentation on acid formation.

The addition of lactic acid had an entirely different effect. Little or no acid was produced by fermentation in this case. The added lactic acid was sufficient to make a final acid content of .7 gram per 100 c.c. without any acid from fermentation and this was approximately the figure reached on the 14th day. In other words, acidity changes in this lot were practically restricted to the dilution of the acid added at the outset. Thus, the addition of lactic acid inhibited the normal production of acid by fermentation.

The curves for brine-sugar concentrations for the same treatments given in the acid curves (Fig. 4) are all similar in shape and

show a rapid rise in the sugar content of the brines during the first three to five days followed by a rapid decrease until about the 18th day, at which time very little sugar remained. The main difference between the curves is the height of the curve for any given day. It is seen that the beginning of the utilization of sugar by fermentation was delayed by the addition of vinegar. The more vinegar added, the longer the delay. Regardless of the delay, once the fermentation started, there was little difference in the rate of disappearance of sugar from the brines of different lots. The difference in the maximum sugar content of the brines does not indicate that there was more sugar in the cases where vinegar was added, but rather that the utilization of sugar did not begin as soon. It is interesting to

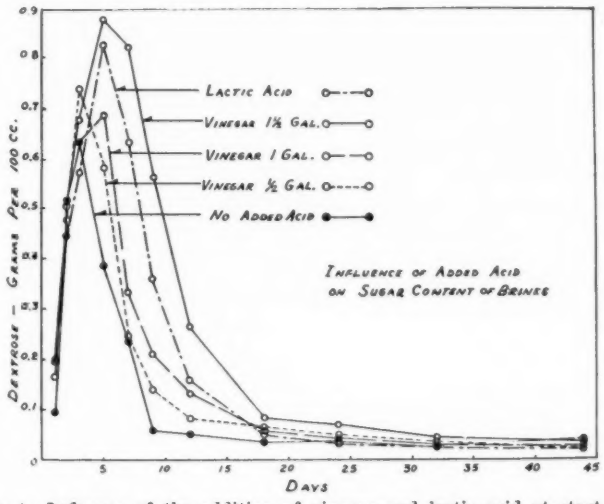

FIG. 4. Influence of the addition of vinegar and lactic acid at start of dill fermentation on disappearance of sugar.

note that the sugar curve for the lot with added lactic acid was similar to the other curves. The sugar was decomposed despite the fact that little or no acid was formed, as was shown by the previous diagram. The sugar was transformed into products other than acids.

The curves for pH for the same lots presented in the last two diagrams (Fig. 5) indicate that there is almost no difference in the values after the first 12 to 15 days; that is, the final pH was independent of the amount of acid added or the acidity developed in the ranges employed in these experiments. The greatest differences appeared during the first 12 days, at which time the main part of the acid formation was taking place. The additions of vinegar reduced

the pH from an initial value of 6.9 to from 3.4 to 4.1 on the first day followed by a slight rise in pH as the vinegar became diluted. After two to five days a decrease in pH occurred owing to the production of acid by fermentation. Successively larger amounts of vinegar caused a longer delay before acid formation by fermentation occurred. The curve for the lot with added lactic acid is obviously unlike the curves for the vinegar series. Apparently the only effect of adding the lactic acid was the rise in pH as the acid became diluted.

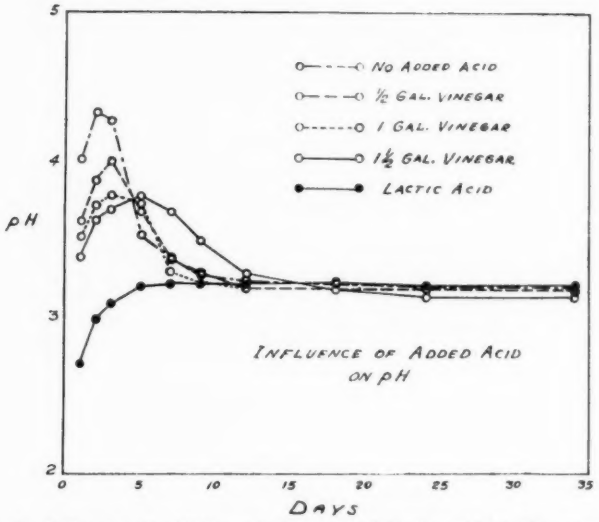

Fig. 5. Influence of addition of vinegar and lactic acid at the start of dill fermentation on brine pH.

The influence of the above-mentioned treatments involving added vinegar and lactic acid (Table 1, Treatments 2, 3, and 5) on microbiological activity during the fermentation is shown (Fig. 6). All treatments were in duplicate and data shown are averages. The results are shown with respect to the predominating microörganisms (upper part) and chemical analyses for reducing sugars and titratable acidity (lower part). It will be noted that the acid-forming bacteria counts reached 100 million per c.c. on the fifth day in the case of the fermentation without added acid and then declined to below the 100,000-per-c.c. range in 30 days. The addition of one gallon of 110-grain vinegar exerted a retarding effect on the lactic fermentation and in general resulted in somewhat lower bacterial

counts. The inhibiting effect of lactic acid (equal to one and three-fourths gallons of vinegar) is evident, the counts being wholly within the 10,000-per-c.c. range. It will be noted also that the fermentation receiving lactic acid showed vigorous activity with respect to yeasts, considerably higher populations than that of the fermenta-

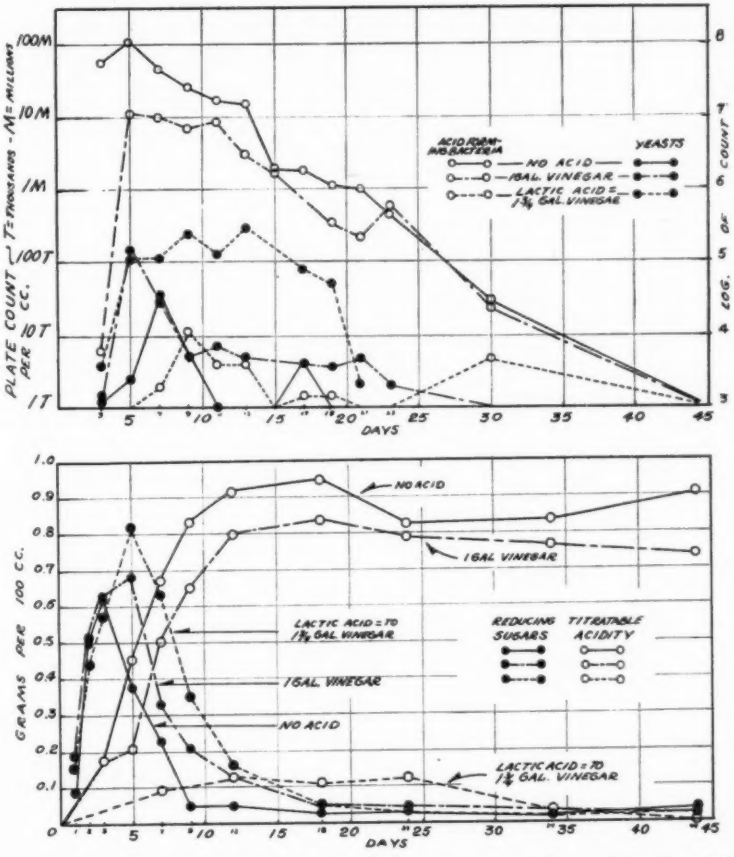

FIG. 6. Growth of predominating microörganisms as influenced by the addition of vinegar and lactic acid at the start of dill fermentation. Upper part, growth curves of acid-forming bacteria and yeasts; lower part, sugar utilization and acid formed by fermentation.

tion receiving one gallon of vinegar. The fermentation receiving no added acid had lower population of yeasts and they appeared for a shorter time than either of the fermentations receiving lactic acid or the one receiving vinegar.

The rate of sugar utilization, as indicated by the decline of the sugar curves, was related to the numbers of the microörganism present in the respective brines at any given time. A sharp decline in sugar content was first observed in the fermentation with no added acid, followed by the fermentation receiving the vinegar.

The lactic acid treatment was the last to show marked sugar utilization. Since there were few acid-forming bacteria in the lactic acid treatment at any time and since the yeast fermentation did not begin vigorous activity until the fifth day, it would be expected that the sugar utilization would be retarded. In the no-acid treatment the fermentation developed approximately .95 per cent acid, while the fermentation receiving one gallon of 110-grain vinegar

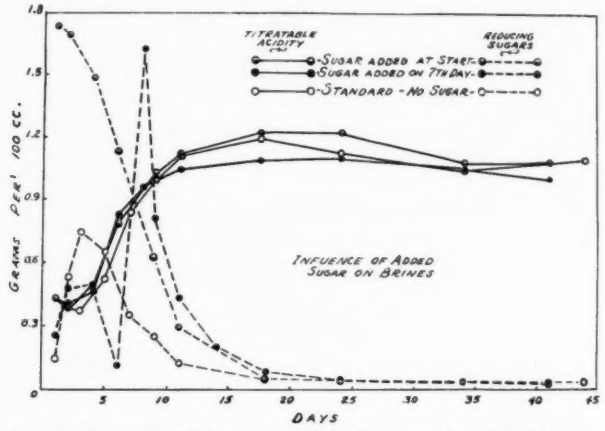

FIG. 7. The effect of added sugar on the acid formation in dill brine.

produced approximately .82 per cent. The lactic acid treatment, which inhibited the growth of acid-forming bacteria, produced approximately .1 per cent acid.

The relationships are shown (Fig. 7) of sugar concentrations and acidity developed in lots receiving standard treatment compared with lots to which three and one-half pounds of sugar were added at the outset and other lots to which the same amount of sugar was added on the seventh day. In spite of the addition of sugar, there was little or no difference in the amounts of acid produced. Lots to which the sugar was added at the outset produced slightly more acid than the standard treatment and the lots which received the sugar on the seventh day produced less acid, but the variations are not much greater than those that would normally be expected within the same treatment. It is particularly interesting to note that the

great differences in the sugar content of the brines from the fifth to the 10th day had little or no effect on the rates of acid formation.

The effect of the addition of three and one-half pounds of sugar at the start of the fermentation on predominating microörganisms is shown (Fig. 8) and the results are compared with another fermentation, started at the same time and under like conditions but having no sugar added. Both fermentations received one-half gallon of 110-grain vinegar as standard treatment. Examination of the acid-forming bacteria growth curves (upper part Fig. 8) shows a sharp

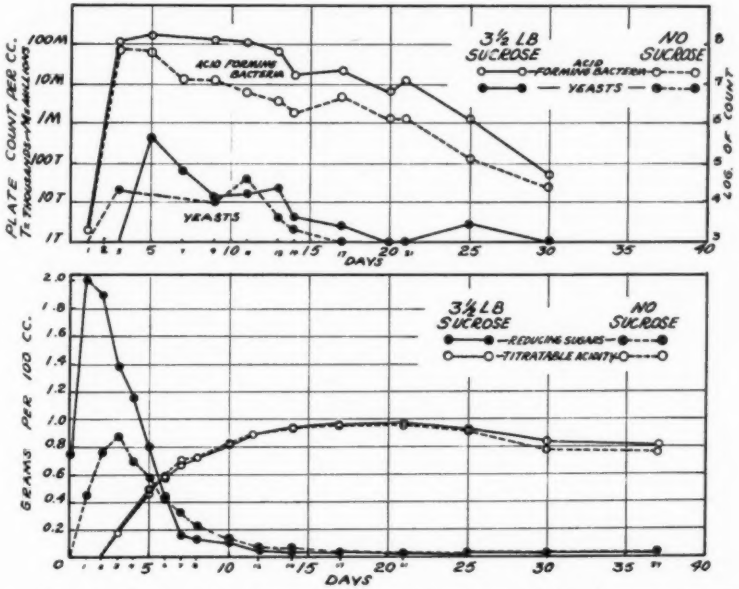

FIG. 8. Effect of the addition of sugar at the start of dill fermentation on growth of predominating microörganisms. Upper part, growth curves of acid-forming bacteria and yeasts; lower part, sugar utilization and acid formation.

rise to 110 millions per c.c. for the fermentation receiving sugar and 70 millions per c.c. for the one receiving no sugar at the five-day plating interval. At this point the growth curves separate and during the major part of the fermentation period the counts from the brine receiving sugar were approximately ten times as great as those from the brine receiving no sugar.

The yeast counts show that in the case where sugar was added the yeast population, in general, was considerably higher and of longer duration than in the case where no sugar was added.

The sugar analyses (lower part Fig. 8) show that in both fermentations the utilization of sugar reached a similar figure after 10 days. It will be noted that there was little or no difference in the amount of total acid produced, indicating that the addition of sugar, while resulting in an approximate ten-fold increase in population of acid-forming bacteria, did not result in corresponding increase in acidity.

A comparison in diagrammatic form is shown (Fig. 9) of all the lots cured according to the schedule outlined in Table 1. In all treatments the brine acidity reached its highest value during the second week of fermentation, and the values for the 18th day generally represented the maximum acidity attained with each treatment.

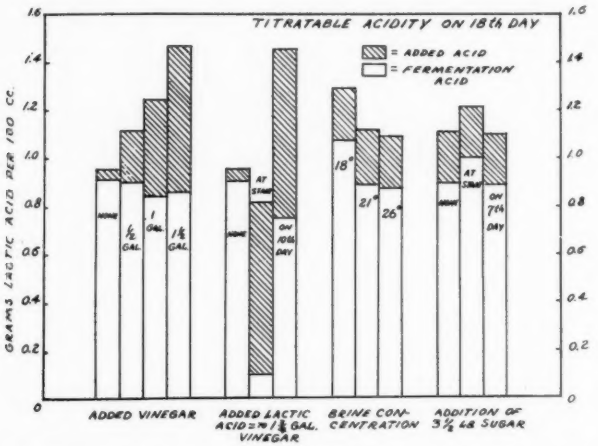

FIG. 9. Summarization of influence of different treatments on amount of acid developed during dill fermentation. Total height of column represents total brine acidity, height of unshaded portion represents acid formed by fermentation.

For the vinegar series this diagram indicates that the addition of vinegar did not materially affect the quantity of acid produced by fermentation.

The values for the lactic acid series demonstrate that the addition of this acid at the outset of the experiment inhibited the production of acid by fermentation processes. However, the addition of lactic acid on the 10th day resulted in a high brine acidity because a normal fermentation had occurred during the period prior to the addition of lactic acid.

The use of brines of different salt concentration resulted in variations in quantity of acid formed. The greater acid production occurred in the brines of lower salt concentration, and lesser acid

production in the brines of higher salt concentration, as shown previously by Jones (1940).

The addition of three and one-half pounds of sugar at the outset of the experiment resulted in an increase in the titratable acidity equivalent to about .1 per cent lactic acid; the addition of the sugar on the seventh day had practically no effect. This indicates that the addition of sugar to the brines did not appreciably increase brine acidity.

The presence of hollow cucumbers or "bloaters" is frequently encountered in the manufacture of dills. In this study some striking differences in the number of bloaters formed during the curing process were observed. Since the cucumbers were large (600 count), the percentage of those found to be hollow was somewhat higher than

TABLE 2

Influence of Some Factors on the Formation of Hollow Pickles or "Bloaters" in Dill-Pickle Manufacture

Treatment	"Bloaters"
	pct.
Standard	20
High salometer (26°)	45
Lactic acid (= 1¾ gal. vinegar) added at start	67
Sugar added at start, 3½ lbs.	78
Sugar added on seventh day, 3½ lbs.	70

might have been expected if cucumbers of the usual dill size had been used. For most treatments the number of bloaters ranged from 10 to 25 per cent. The treatments which favored the development of bloaters in considerably greater numbers than the standard are shown (Table 2).

SUMMARY AND CONCLUSIONS

Various methods of manufacture of dill pickles under southern conditions have been tested. The effects of brine salinity and the addition of vinegar, lactic acid, and sugar upon fermentation were determined by chemical and bacteriological analyses of the brine during the curing process. Observations of the influence of some of these factors on "bloater" formation have been made. The results of these studies may be summarized according to the various treatments as follows:

Vinegar added at the start of the fermentation delayed acid formation but did not appreciably influence the quantity of acid produced or the rate of disappearance of sugar from the brine.

The addition of lactic acid inhibited the normal production of acid but did not markedly affect the rate of sugar utilization. Lots

receiving lactic acid at the outset of the experiment gave high percentages of bloaters while those lots receiving the acid after 10 days gave moderate percentages of bloaters.

The addition of sugar did not significantly increase the production of acid by fermentation, although there was a rapid utilization of sugar. However, the addition of sugar resulted in a marked increase in the percentage of bloaters formed.

Increases in brine salinity resulted in slightly decreased total acid formation but did not otherwise markedly affect the production of acid or utilization of sugar. However, brine concentrations much above 21° salometer are not usually satisfactory since the finished product is too salty for edible purposes.

Under the conditions which prevailed during the course of these experiments the predominating microörganisms in dill-pickle fermentations were found to be acid-forming bacteria and yeasts. Peptonizing bacteria were also recognized in the flora of the dill fermentation. These latter appear in relatively small numbers, however, and may be wholly absent, particularly in cases where acid is added at the start of the fermentation.

The acid-forming bacteria were present in the greatest numbers, millions per cubic centimeter, within two to four days and remained in high counts for approximately two weeks. Following this period their numbers decreased although they were present in the brine for about 30 days.

The yeast fermentation was found to be common to all dill fermentations studied. This is of particular significance inasmuch as the definite rôle of yeasts in dill fermentations has been, heretofore, generally unrecognized. The yeast fermentation occurred after the acid fermentation had reached a definite activity. It was more irregular in its course, in some instances, than the acid fermentation. As indicated by plate counts the yeasts are present in decidedly lower numbers per cubic centimeter than acid-forming bacteria.

It was found that the addition of sugar to a dill fermentation resulted in a marked increase in population of acid-forming bacteria, with no appreciable increase in final acidity. Also, the yeast population was somewhat higher in the fermentation to which sugar was added.

The addition of one gallon of 110-grain vinegar to a dill fermentation showed an inhibiting effect upon the population of acid-forming bacteria but did not significantly influence the quantity of acid produced by fermentation as compared with a treatment without added vinegar. When vinegar was added, the yeast fermentation started sooner and showed moderately higher counts than a comparable fermentation without vinegar.

A marked inhibition of the acid-forming bacteria was evidenced by the addition of lactic acid to a dill fermentation. The counts of these organisms were found to be decidedly lower than in the case where added vinegar was employed. The use of lactic acid resulted in a most active yeast fermentation of prolonged duration, accompanied by high counts as compared with other yeast fermentations.

ACKNOWLEDGMENT

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EDITORIAL REVIEW

COMMENTS ON THE MICROBIOLOGY OF SPOILAGE IN CANNED FOODS[1]

E. J. CAMERON AND J. R. ESTY
Research Laboratories, National Canners Association, Washington, D. C., and San Francisco, California

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These comments on the microbiological aspects of canned-food spoilage are rather specifically based on the experience of our laboratories and they outline our existing concepts of the microbiology of canned-food spoilage. No attempt is made here to provide a review of the extensive literature on the subject. Particularly we desire to emphasize the significant peculiarities of the problem as we view them.

The laboratories which the authors represent are located in Washington, D. C., and San Francisco. Both center interest on canning of fruits and vegetables. In the East the latter receives primary consideration; in the West there has been greater opportunity for study of fruit problems. Study of canning problems at these two points have established the conclusion that for the most part spoilage experience is in agreement.

In one respect, and we believe that this is a point which has not been emphasized properly, spoilage in canned foods is unique. Three groups of thermophilic organisms are economically the most important causes of spoilage in low-acid vegetables. So far as we know they are not of importance otherwise in food spoilage. Their importance in canning rests largely on the high resistance of their spores. Other bacterial types causing spoilage in canned foods, such as the lactic acid bacteria, possess wider economic significance and have been subjected to study by research workers interested in unpreserved foods and in fermentation. It has remained to those interested in canning problems, therefore, to give specialized attention to these three thermophilic groups, not only from the standpoint of taxonomy but also the study of their spoilage habits, sources, and practical means of control. It is noteworthy in this connection that only in recent years have the classification systems made any reference to these important bacteria. Such thermophilic spore-formers

[1] Presented before the Third International Congress for Microbiology, Section VIII, Subsection II, September 5, 1939, New York, N. Y.

as were described fell into that segment of the aerobic spore-forming group which exhibits rather amazing abilities to temperature adaptation. As a practical matter these aerobic types are not of remarkable importance to canning problems.

THERMOPHILIC SPOILAGE BACTERIA

There is no place in this review for extended description of the spoilage bacteria. In view of the peculiar importance of these three thermophilic groups, however, reference is made to their principal distinguishing characteristics.

(1) The flat-sour bacteria are so called because they cause spoilage through acid production, without gas. The type species is *Bacillus stearothermophilus* and was described by Donk (1920). Subsequent studies were made by Cameron and Esty (1926). These bacteria are probably the most noteworthy economically because they are facultative anaerobes and their ability to grow under high or low oxygen levels is responsible, in large part at least, for their prevalence as noted in contamination sources which have been defined.

(2) There are two groups of thermophilic anaerobes, the more important of which does not produce H_2S. These may be rated as second in importance to the flat-sour group. Their spores are of high resistance to heat and they are of especial importance in products of intermediate acidity. For many years these organisms were recognized principally by their high-resistant spores, anaerobic-growth requirements, and ability to grow at high temperature. It was known that the gas which they produced consisted of hydrogen and CO_2, and the odor which resulted when growth occurred was suggestive of butyric acid. The absence in the literature of reference to such organisms led to more than one expression of doubt that they existed. The significant work of McClung (1935), however, has defined clearly the position of this group in the system of bacteriology and he has named the species *Clostridium thermosaccharolyticum.*

(3) The third position in this group of thermophiles is allocated to *Clostridium nigrificans*, described by Werkman and Weaver (1927). The property of H_2S production and the resultant change in canned products affected by it has led to the more homely designation "sulfide-spoilage" bacteria. The organism is not of great importance commercially, but has caused heavy spoilage in a few instances.

A newcomer to the group of thermophilic bacteria of importance in canning is *Bacillus thermoacidurans*, Berry (1933). This is closely associated with the flat-sour group to which reference has been made and shows an unusual ability to grow in products of relatively low pH. Noteworthy, also, is a materially lower spore resistance than

is associated with the flat-sour bacteria which cause spoilage in the low-acid products. This organism confronted the industry with the advent of commercial canning of tomato juice.

ACIDITY CLASSIFICATION OF SPOILAGE TYPES

Spoilage relationships in canned foods are associated with the factors of nutrients, temperature, moisture, salt content, and the like, but the best definition of types appears to rest upon an acidity classification of the materials affected. In an earlier paper on the microbiology of canning, by Bigelow and Cameron (1932), such a classification was the subject of discussion and an arbitrary acidity grouping was suggested. There was recognition of the fact that, strictly speaking, all vegetables and fruits are acid to some degree, but for convenience the three primary groups were referred to as nonacid, semiacid, and acid. The upper limit of the acid range was placed at pH 4.5, and the semiacid at pH 6. Experience derived since the publication of that statement has indicated some modification with respect to acidity levels.

The improved scheme of classification would place a low pH limit at 4.5 for a principal acidity group, subdivided to include low-acid products with a lower limit at about 5, Group 1, and those of medium acidity, Group 2, pH 5 to 4.5. Below pH 4.5 a further division is warranted at about pH 3.7, forming Groups 3 and 4. The reasons for these groupings are as follows:

Group 1—Low Acid—pH 5 and Higher: In this group fall most meat products, the marine products, milk, and certain of the common vegetables, such as corn, peas, lima beans, asparagus, and spinach. All of them are subject to spoilage by the three principal thermophilic groups which have been described, although these organisms are not the usual cause of spoilage in meat and marine products. There is the exception also that the "sulfide-spoilage" bacteria have not been important in products having a pH toward the lower level of the low-acid range. The mesophilic group of putrefactive anaerobes is of importance in spoilage of all these products and is the principal cause of spoilage in meats. Of materially lesser importance are the so-called aerobic spore-formers and a number of odd types of which *Bacillus betanigrificans*—Cameron, Esty, and Williams (1936)— is an example.

Group 2—Medium Acid—pH 5 to 4.5: This in effect is a subdivision of Group 1. It contains principally meat and vegetable mixtures and "specialties," such as spaghetti, soups, and sauces, whose ingredients blend to partial acidity. The organisms referred to in Group 1 may be of importance in Group 2, the principal reason

for differentiation being that the thermophilic anaerobes (not producing H_2S) are of increased importance in relation to the flat-sour group. Also, in general, the flat-sour organisms capable of development in this median environment appear for some reason to be lower in resistance than the most resistant of those causing spoilage in the products of lower acidity. In some products of this group, also, the putrefactive anaerobes may show abnormal growth, resulting in non-gaseous changes in the food.

Group 3—Acid—pH 4.5 to 3.7: This group contains tomatoes, pears, figs, pineapples, and nectarines as well as other fruits. It is particularly important through the fact that the products which it contains are subject to spoilage not only by nonsporing, aciduric types but also spore-forming anaerobes which are allied in their characteristics to *Clostridium pasteurianum*, Winogradsky. Spoilage by these butyric anaerobes has been studied by Townsend (1939) on the West coast. Strangely, this type of spoilage has not been encountered in the East in tomatoes, which is the product packed in large quantities common to the two sections. Townsend found that contamination occurred in the field rather than in the cannery and that the organisms appeared to be present in the soil of different districts in large numbers. In an earlier publication Townsend (1929) provides a classification of acid-tolerant bacteria causing spoilage in canned foods. For these acid products he notes spoilage through the activity of cocco-bacilli growing at 30 and 37°C.(86 and 98.6°F.). He notes also the occurrence of spore-forming, flat-sour types growing at 30°C., and clostridia growing at 37°C. These classes are described by him in detail. An experience peculiar to the West has been the spoilage in this acidity range caused by thermophilic anaerobes.

Pederson (1929) has comprehensively studied bacterial spoilage of tomato products and his report constitutes the most valuable contribution in this field. He reported on the examination of 266 cultures of bacteria obtained from 83 samples of tomato products. All but a few miscellaneous cultures were classified as belonging to six species, of which all but one produced gas in glucose broth. It is probable that the organisms identified by Pederson contain those responsible for the great majority of cases of spoilage in tomato products, excepting tomato juice. All these organisms are nonspore-formers and controllable by processes at 100°C.(212°F.).

Spoilage in tomato juice constitutes a unique problem among tomato products in that the principal cause of spoilage in the East is *Bacillus thermoacidurans*, Berry (1933). Prior to the canning of tomato juice on a commercial scale such spoilage in tomato products

was unrecognized. The origin of this organism undoubtedly is in the soil, but it has been found that a concentration of contamination may develop in certain types of equipment, and control measures have been based upon knowledge of where such contamination is likely to occur.

Before leaving Group 3 a note should be made that products within its scope are for the most part processed at 212°F. The occurrence of spoilage from spore-forming organisms in this group creates a special problem which apparently must be dealt with from the standpoint of contamination control rather than process increases. Indeed, it is difficult to say just what processes are necessary in order to dispose of such contamination.

Group 4—High Acid—pH 3.7 and Below: This group, distinguishable from Group 3 principally through the fact that commercial experience has indicated freedom from spoilage caused by spore-forming bacteria, contains products such as kraut, pickles, berries, grapefruit, citrus juices, and rhubarb. Here we find little in the way of restriction as to nonsporing, microbiological agents causing spoilage. Aciduric, nonsporing bacteria are important and undoubtedly the organisms listed by Pederson (1929) are of significance. With respect to these organisms, it is interesting to note that certain of them were studied some years ago in our laboratories with respect to spoilage habits in different fruits. It was found that acidity of itself was not the controlling factor. Spoilage did not occur in pigmented fruits, such as blueberries and loganberries, although the organisms would cause spoilage in unpigmented products of higher acidity. Our experiments on the inhibitory effects of fruit pigments were not comprehensive and no report was published. It is believed, however, that considerable interest would attach to an extension of such work.

YEASTS AND MOLDS

In addition to the aciduric organisms causing spoilage in this group we must include yeasts and common molds. Yeasts are infrequent causes of spoilage in canned fruits and there is no reliable information as to the extent of damage caused by them. In all likelihood the amount of loss is small. In recent years remarkably few samples of yeast spoilage, or reports of such spoilage, have come to our laboratories.

Molds carry considerable importance with respect to the condition of the product before canning, but they are not causes of spoilage after canning so far as experience in this country is concerned.

We have recently concluded a study of mold growth in canned berries which constitutes a unique experience and will be the sub-

ject of a published report in the near future. Passing mention of it is warranted here. Last year it was called to our attention that in some large cans of canned berries small patches of mold appeared in the headspace of the can in spite of the fact that these berries had been brought to a temperature of well over 87.8°C.(190°F.) in the canning process, and some of the cans in which these patches appeared carried vacuums as high as 18 inches. It thus appeared that this organism must possess unusual resistance to heat and ability to grow under reduced oxygen. Laboratory studies of this mold indicate for the sclerotia a resistance in berry juice of greater than 200 minutes at 82.2°C.(180°F.), and some growth has occurred with the vacuum higher than 25 inches. This mold is not to be regarded as a spoilage type because any effect produced by it under the conditions we have cited is so limited as not to be detectable other than through the occurrence of small mold patches. However, this does illustrate the anomalous conditions encountered from time to time in acid products.

ACIDITY AND GROWTH OF *CLOSTRIDIUM BOTULINUM*

Following this more or less detailed discussion of the spoilage groups we wish to call attention once more to the important relationship between acidity and possible growth of *Clostridium botulinum*, which is the only spore-forming organism of demonstrated importance in the healthfulness of canned foods. This is a subject which has been discussed on many occasions, but no paper of this kind would be complete without further reference to it.

For many years laboratories connected with the canning industry, and other laboratories, have devoted attention to *Clostridium botulinum*. Upon the industry itself has devolved the task of developing information which could be applied in a practical way to control of the organism. A great amount of work has centered on heat-resistance studies, and processing recommendations for low-acid foods are based upon the results of this research. Concurrently a study was made of the growth necessities for *Clostridium botulinum* and it was found that the dividing line of acidity between products in which the organism would grow and those in which it would not grow was about pH 4.5. This level is considered inhibitory for the growth of the organism in a favorable medium. Under certain conditions, as where there is low nutrient, growth may be inhibited regardless of pH. This is a most important observation because as a practical matter it means that, in general, products of pH levels higher than pH 4.5 must be processed under pressure in order to insure destruction of the spores, while products at pH 4.5 or lower may be safely proc-

essed in the open bath, 100°C.(212°F.). The experience of the industry over a spread of many years has established the validity of this product classification.

The industry has given emphasized attention to spoilage control and much has been published in connection with the so-called "processing studies" which were set in motion by our late chief, Dr. W. D. Bigelow, and which are still in progress. Two salient bywords have been associated with this work—"safe processing" and "contamination control." With regard to the former, processes for nonacid (low-acid) products have been recommended in bulletin form to the industry since 1930. The present processing bulletin, National Canners Association Bulletin 26-L (1939), is the fourth edition, and processes given in it represent the composite opinion of experts in canning technology. In the main, the processes are based upon technological data relating to the heat resistance of spoilage bacteria in canned foods and upon data pertaining to heat penetration in canned foods. In keeping up to date with processing information, periodic conferences are held by representatives of laboratories connected with the canning industry. At these meetings information is pooled and decisions made with respect to changes in recommendations or additions to the products which are included.

In the matter of contamination control much is now known about significant sources of spoilage contamination and its control, and essential information has been carried to the industry by means of publications and addresses at canners' meetings. It has been emphasized that, in general, the use of wood in canning equipment has been hazardous, for bacteria may become seeded in the pores and once established may contaminate food materials to such an extent that spoilage may occur with a process that has been satisfactory for years. It has been brought to attention also that pumps, pipes, extractors, and other elements of metal equipment should be selected from the standpoint of ease in cleaning.

In recent years special attention has been devoted to studies of contamination of ingredients used in canning. Much information has been developed which can be applied directly in reducing spoilage losses. Among the ingredients which may be important carriers of spoilage contamination are sugar, starch, flour, and dried milk. Of these, sugar and starch are most important. Recent developments in canning technology having reference to spoilage control received consideration at the Food Technology Conference held at the Massachusetts Institute of Technology in 1937, reported by Cameron (1938).

SPOILAGE RESULTING FROM CAN LEAKAGE

In order not be accused of an important omission, our final comments concern spoilage which results, not from under-sterilization, but from can leakage after processing. This phase of the subject is more important from the mechanical than from the biological standpoint. The organisms which cause spoilage in the event of leakage are the heterogeneous free-living forms not subject to orderly classification in relation to canned-food groups. There is, however, an important biological differentiation between spoilage from under-sterilization and from leakage, as in the case of low-acid foods. In this group when spoilage occurs from under-sterilization it is due usually to a single spore-forming type. Where leakage is concerned, on the contrary, it is customary to find mixed cultures of nonsporing bacteria which could not have survived the process and which, therefore, must have entered the can after the process. This differentiation does not exist in a clean-cut way in the case of the acid products because the aciduric organisms which cause spoilage may have been present in the product at the time of canning or may have entered subsequent to the process. Some guidance as to the cause of spoilage, however, may be had from observations as to whether spoilage occurred from one or a few or many bacterial types. In the latter instance leakage is indicated.

As stated in the early part of this paper, these comments are not meant to be comprehensive. It has been possible only to outline our experience in the spoilage of canned foods and the relationships which appear to have fundamental importance. Expansion of the observations made here are, for the most part, to be found in the scientific literature on canning.

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COMPOSITION OF FIREWEED HONEY

JOHN A. ELLEGOOD AND LOUIS FISCHER
College of Pharmacy, University of Washington, Seattle, Washington

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In the Pacific Northwest a wide variety of honeys are produced, many of which are common to all honey-producing districts in the United States. In the Puget Sound region, a very desirable and unusual honey, known as "fireweed," constitutes a considerable portion of the annual harvest. This kind is not so widespread in its occurrence as other common types but is becoming increasingly popular because of its pleasing flavor. The fireweed honey sold on the market may range from nearly colorless to amber in color and frequently varies in taste from a mild to a more pronounced characteristic flavor.

Frequently samples of fireweed honey are submitted by proper authorities for analysis. Since no reports for authentic samples could be found, it was thought advisable and of considerable value to obtain pure samples and to determine their approximate composition and physical and chemical constants for use in future identification of fireweed honey submitted for analysis in various food and drug laboratories.

DESCRIPTION AND HABITAT OF FIREWEED

Epilobium angustifolium is the commonest of several species of fireweed found in the Northwest. The plant is perennial, averaging about one meter in height, with numerous glabrous or puberulent, lanceolate, nearly sessile leaves. The flowers are nearly always rose-colored and arranged in a raceme.

The plant is common in open woods and along roadsides and is especially abundant in freshly burned over or logged-off land. It is the first of a succession of regrowth, being readily crowded out by the vigorous succeeding ferns, blackberries, and other wild growth.

COLLECTION OF SAMPLES

The difficulties encountered in the collection of a honey from a particular kind of flower are quite obvious. The apiarist usually places his hives in areas where there is an abundance of flowers, and the name of the honey may be taken from the most prominent flowers in bloom. In the western part of the state of Washington, fireweed blooms usually from the early part of July to about the middle of August, a time when few other flowers are in blossom. Therefore,

TABLE 1

Polarizations and Analyses of Fireweed Honey

Sample	Polarization					Complete analysis						Free acid as formic
	Direct			Invert								
	Immediate at 20°C. (68°F.)	Constant at 20°C.	87°C. (188.6°F.)	20°C.	87°C.	Water	Invert sugar	Sucrose	Ash	Dextrin	Undetermined	
	°V.	°V.	°V.	°V.	°V.	pct.	pct.	pct.	pct.	pct.	pct.	pct.
A	— 9.2	—11.0	15.10	—15.3	0.90	15.50	69.85	4.52	.04	.49	9.60	.04
B	—14.5	—17.3	7.30	—12.7	1.20	13.91	74.62	2.65	.04	.65	8.13	.05
C	—13.8	—14.5	9.62	—24.2	3.06	15.83	70.92	8.50	.04	.78	3.93	.03
D	—16.3	—16.6	7.89	—24.8	2.40	17.56	70.73	7.40	.05	.99	3.27	.04

if the proper precautions are taken, a product can be obtained which has as its source, for the greater part, the fireweed bloom.

Sample A was obtained from Mr. Chester A. Brown, the state bee inspector of the northwest district for the state of Washington. The bees were moved into a district of logged-off land, near Carnation, Washington, in which there was an abundance of fireweed and but few other flowers at this time of year. The bees were allowed to fill completely the empty frames in the supers during the peak of the bloom, which was between July 18, and August 18, 1938. The supers were then removed and taken immediately to the laboratory for analysis.

Sample B was collected in a similar manner from a district near Enumclaw, Washington, during the 1939 season.

Sample C was obtained from an apiarist near Bothell, Washington, in 1938, and it was said to be pure fireweed honey.

Sample D was purchased on the open market in the fall of 1938, and was labeled fireweed honey.

EXPERIMENTAL PROCEDURE

The comb samples, A and B, were cut across the face and placed on several thicknesses of cheesecloth over large beakers and allowed to drain. The strained honey was then transferred to glass jars and sealed. Samples C and D had been strained before the time of purchase and required no further preparation.

The procedures outlined under honey in the methods of analysis of the Association of Official Agricultural Chemists[1] were used for each determination. In the determination of sucrose the amount of naturally occurring invert sugar was subtracted from the invert sugar obtained after inversion of the sucrose with hydrochloric acid (sp. gr. 1.1029 at 20/4°) and the difference was multiplied by .95. In each case the invert sugar was determined using the Munson and Walker method for the estimation of reducing sugars. All analyses were made in duplicate; the results were averaged and are presented (Table 1).

SUMMARY

Data for direct and invert polarizations at 20 and 87°C.(68 and 188.6°F.) and the results of other analyses—water, invert sugar, sucrose, ash, dextrin, and free acid calculated as formic—are given for four samples of fireweed honey. The results of the above determinations for each of the four samples vary over a slight range, but they give definite information as to the composition and constants of fireweed honey.

[1] Association of Official Agricultural Chemists, 1935. Fourth ed. Official and Tentative Methods of Analysis, 487-490.

A STUDY OF STARCH FROM DIFFERENT VARIETIES AND TYPES OF CORN

LOUISE PICKENS TANNER AND D. T. ENGLIS
Noyes Laboratory of Chemistry, University of Illinois, Urbana, Illinois

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Starches from different plant sources, though unlike in microscopic appearance, chemical constitution, and practical working properties, are so similar in behavior that all are classed as starch. The chemistry of starch has been such a fertile field for investigation that the literature is voluminous and, in some respects, quite contradictory. Although some of it is valuable in showing the development of present theories concerning starch structure and properties, much of it is of little more than historical interest. Many starch investigators have reported two modifications within the starch granules although they do not agree as to what constitutes these differences between components. Some attribute them to configuration differences, others to presence or absence of non-carbohydrate materials.

It is generally agreed that the fundamental unit of starch is constructed from 1-4 glucopyranose residues united in a chain-like fashion. Whether these units are relatively short chains, large rings, or long, straight, or branched chains has not yet been determined, though much thought and work has been focused on the problem by such chemists as Haworth, Freudenberg, and Staudinger.

Small amounts of non-carbohydrate materials are believed to be esterified with the carbohydrate residues. Many starches contain phosphates and some have fatty acids. Minerals occur in the ash from starch. These may be derived from impurities acquired during manufacture, from adsorption complexes, or from integral parts of starch molecules.

These factors, chemical make-up of the carbohydrates and amount and kind of non-carbohydrates associated with them, are responsible for different properties of starches from various plant sources. Numerous tests have been applied to starch materials in an effort to characterize them as to source; certain limits of variation have been found to exist for the starch obtained from certain species of plants.

The desire of plant breeders to develop plants of greater disease resistance and crop yield or to meet the demands of some manufacturer has resulted in an extension of the limits of ordinary variation; for example, at the University of Illinois during the past 40 years,

four strains of corn containing entirely different amounts of fat and protein have been developed from the same parent strain. The 1938 percentages were as follows: high protein, 17.5 per cent; low protein, 5.8 per cent; high fat, 13.1 per cent; and low fat, 1.3 per cent. During these years the quantity as well as the quality of starch in the corn kernels underwent changes. Corn kernels contain from about 86 to 93 per cent of starch. This starch may be hard or soft. "Dent" corn, the variety usually grown in the Corn Belt, may contain various ratios of hard to soft starch. Horny and waxy kernels contain relatively large amounts of hard starch. Floury types are largely soft starch.

The development of new strains of corn, not only of widely different composition but of apparently different character of starch itself has given rise to new problems in commercial utilization of corn. Corn starch manufacturers and users of corn for whiskey production have observed that lots from different localities do not have the same response to processing and are apparently unlike in many respects.

Most studies of chemical and physical properties of corn starch have been carried out on commercial samples which were undoubtedly a mixture derived from many kinds of corn. It seemed advisable to have information upon samples which were known to have been prepared in each case from a single variety. The purposes of this investigation were (1) *to isolate starch* from as widely different varieties and types of corn as could be obtained; (2) *to examine each sample* as to the form of granule and the amount of non-carbohydrate constituents associated with the starch; (3) *to study the swelling power*, viscosity, and other factors associated with the physical characteristics of starch; (4) *to determine any significant difference* in the carbohydrates as manifested by a separation of the starches into alpha-amylose and beta-amylose fractions by methods described by Taylor and his associates, and (5) *to characterize* these fractions by a study of their properties.

ISOLATION OF CORN STARCH AND ANALYSIS FOR NON-CARBOHYDRATE CONSTITUENTS

In order to study the properties of corn starch, it is desirable to have the starch retain, as nearly as possible, its native characteristics. Lamm (1937) suggested that starch, during its separation from other constituents in plants and its subsequent purification, may undergo changes which are analogous to denaturization of protein. Taylor and Iddles (1926), Samec (1936), Woodruff and MacMasters (1936), and Gallay (1937) have described differences in starch behavior following treatment with heat, water, acids, and alkalies

which indicate that some constitutional changes had taken place within the starch molecules. The rate of precipitation of starch from aqueous solution by alcohol as it influenced the X-ray pattern of the precipitated material was observed by Samec and Katz (1937). Alcohol, thus, may cause molecular rearrangements in starch units, though these may conceivably be of a reversible nature. The action of alcohol upon unswollen starch granules is not comparable to the precipitation of starch from aqueous solutions. In the former case no change appears in the starch, but in the latter a characteristic product, known as alcohol-retrograded starch, is obtained.

Starch manufacturers steep corn from 30 to 40 hours in water containing .25 to .3 per cent sulfur dioxide. In order to avoid an acid treatment and in the hope that these solutions were less damaging to native starch, Alsberg and Rask (1924) washed starch from wheat dough and purified the starch by treating it with successive portions of sodium chloride, water, alcohol and ether to remove globulins, sodium chloride, alcohol-soluble and ether-soluble materials, respectively, from the impure starch. Woodruff and Hayden (1936) adapted the method of Alsberg and Rask to corn starch purification.

Different characteristics of starch have been ascribed to the presence of small amounts of non-carbohydrates. Since starch is separated mechanically from other substances with which it occurs in plants, it is probable that the separation is never complete. Pringsheim (1932) stated that pure starch can be obtained only with the greatest difficulty and great loss of material.

In an attempt to characterize the seven varieties of starches studied, analyses were made for moisture, total ash, nitrogen, phosphorus and fatty acids. Titration and pH determinations showed the acidity of the specimens.

Because of its hygroscopic nature, Ripperton (1931) and Katz and Derksen (1937) believed it advisable to work with starch of known moisture content rather than with anhydrous starch.

The ash of corn starch represents both organically bound elements and impurities. It may be largely phosphorus and any inorganic cations may be associated with phosphoric or fatty acids attached to the polysaccharide units. Traces of aluminum, chlorides, sulfates, iron, and silicon have been reported. Ling and Nanji (1925) suggested that silicon might occur as a calcium-magnesium salt of a silicic acid-phosphoric ester of amylose. Malfitano and Catoire (1928) described polymers of calcium-potassium-phosphoric esters of amylose; from .03 to .084 per cent of ash has been reported in corn starch analysis.

Another controversial question relates to the condition of nitrogen found in corn starch analysis. Most investigators have considered it an impurity. Samec (1932) believed it a constituent of what he termed "amylocellulose." He considered it important in relation to electrochemical properties, and thought that small amounts of it cause significant changes. Koets (1936) concluded that amylopectin is probably a flocculated coacervate of amylo-phosphoric acid and one or more protein.

The phosphorus content of potato starch and its influence on starch behavior have been subjects of numerous investigations. The experiments of Samec (1936) led him to relate paste formation to esterification of amyloses with phosphoric acid. These esterified amyloses probably account for the gelatinous condition of potato and canna pastes. Wheat starch pastes owe their properties to the presence of nitrogen with phosphoric acid. In corn starch fatty acids are said to replace part of the phosphorus and to produce "pasty" instead of "pectinous" pastes. According to Küntzel and Doehner (1939), it is unlikely that the phosphate content of starch varies from one part of the granule to another, as Samec has postulated. Edwards and Ripperton (1933) found corn starch low in phosphates as compared with potato starch. Posternak (1935) decomposed corn starch by acid and enzyme hydrolysis and concluded that phosphorus was only loosely combined with the carbohydrate. His experiments with potato, arrowroot, and sago starches agree with current opinion that in these starches phosphorus is closely combined with polysaccharide units. It is unfortunate that the cereal starches have been studied so little and that the generalizations so frequently encountered in starch literature arise from investigations of non-cereal, and especially potato, starches.

Until the time of Taylor and Nelson (1920) any fat associated with corn starch was assumed to be due to contamination which could be removed by solvents. Taylor and Nelson, however, were unable to remove all fats from commercial or treated starches and reported that the acids so combined could be removed only after hydrolysis. They concluded that the fatty acids could not be attributed to retention of fat between layers in starch granules. The researches of Taylor and his coworkers (1926-1936) substantiated the idea that fatty acids are an integral part of the carbohydrate complex. Taylor and Werntz (1927) attributed the polarity of the alpha-amylose fraction of corn starch to its fatty-acid component since polarity disappeared upon removal, by hydrolysis, of fatty acids. Recently Schoch (1938), a former coworker of Taylor, re-

moved fats from starch by the use of water-miscible fat solvents and concluded that fatty acids are distributed throughout the granules as extraneous impurities. Lehrman (1939) questioned Schoch's interpretation, but did admit that fat may exist as an adsorption complex with carbohydrate. Such a condition could prevail and produce results such as Taylor and his associates obtained. Thus, the question is open again.

Snider and Coleman (1937) stated that the determination of acidity of starch by means of titration methods is of no value since almost any value can be obtained depending upon the indicator used.

Hydrogen-ion concentration has usually been determined colorimetrically. Grimshaw (1928) centrifuged a suspension and determined the pH of the clear liquid. Ripperton (1931) experienced difficulties with this method and worked with stirred suspensions. He believed that only a small part of the phosphate ion entered the water extract; his experiment showed that the pH of suspensions was always two- or three-tenths of a pH lower than that of the corresponding water extracts.

Experimental Procedure

The varieties of Illinois corn used in this experiment were Pride of Saline, Sutton, Golden Beauty, and h. h. Part of the Sutton variety, designated as Sample 11, was frozen on the cob one week after harvesting. Hard starch granules predominated in the first three varieties in decreasing amounts. Variety h.h. was a floury corn with soft starch predominating. Starch was prepared from these five samples and from one variety each from Argentina and Africa, according to the technics of Woodruff and Hayden (1936).

The amounts of moisture, ash, nitrogen, and fat (acid hydrolysis) associated with the corn starch samples were determined by the methods found in "Official and Tentative Methods of Analysis" of the Association of Official Agricultural Chemists (1935). Phosphorus determinations were made according to Meigs, Blatherwick, and Cary (1919). The pH of starch suspensions prepared according to the suggestion of Ripperton (1931) were read on a Coleman pH electrometer.

Acidity was determined by titrating 20 grams of starch in 100 ml. of redistilled water, after the starch-water suspension had been stirred continuously for 10 minutes, with tenth-normal sodium hydroxide until the solution was faintly red to phenolphthalein.

Discussion of Results

Starches made from seven samples of corn looked alike to the unaided eye and felt the same when pressed between the fingers. Microscopic examinations revealed granules of similar sizes and shapes in all starches. The differences between starches were in the ratios of large, round granules to small, irregular-shaped granules. Photomicrographs indicated that more small granules were to be found in hard than in soft varieties of corn. These differences may be observed (Fig. 1) in photomicrographs of starches made from the hardest and softest varieties of Illinois corn. These findings are

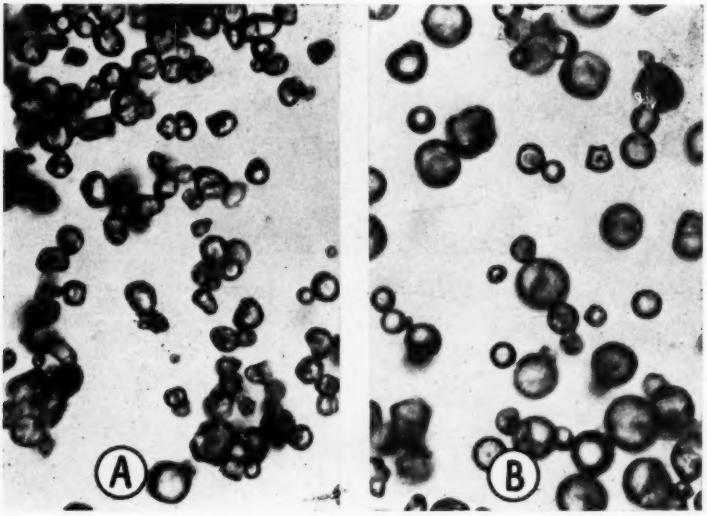

FIG. 1. A. Granules of starch made from Illinois hard corn, Pride of Saline. B. Granules of starch made from Illinois soft corn, h. h. (magnification about ×400).

in keeping with the observations of Brink and Abegg (1926) and Buchanan and Naudain (1923) that size and hardness are related. Granules of hard Argentina corn starch were slightly larger and less rounded than those of medium hard Illinois starch, while soft African corn-starch granules were quite similar to Argentina corn-starch granules. These findings illustrate the indefiniteness of connotation given the word "hard" in different localities.

In the microscope field of one sample of starch there appeared an irregular-shaped mass. Later analysis of this starch showed that it was not as completely freed during purification from protein materials as were the other samples.

Radial cracks were noticed in the starch granules from corn which had been frozen on the cob for one week immediately after harvesting. Some of the granules of this starch were slightly larger than granules from the corresponding unfrozen corn. This observation and subsequent behavior of the starch tend to corroborate the statement that starch from frozen corn takes on characteristics of a softer starch.

The analyses of non-carbohydrate constituents together with a characterization of the starch samples are recorded (Table 1). The numbers indicating percentage of moisture associated with corn starch are an average of two determinations, one made at the beginning of the study, the other made about six months later. In every case moisture had decreased slightly during this period. The decrease does not seem to be a function of the hardness of corn starch.

Larger amounts of ash were obtained from the harder Illinois starches. The low ash obtained from starch from frozen corn suggests that freezing changed the character or binding of the mineral constituents in such a way that some were lost during preparation of the starch. Numerically, the difference is small though it represents a 25-per cent loss. The amounts of ash found in the Illinois samples of corn starch agree quite well with other published results.

The amounts of phosphorus listed (Table 1) range between high and low published amounts. Lower percentages were found in the soft starches, correlating with the total ash constituents. The reduced amount, about 50 per cent. of phosphorus obtained from frozen corn starch adds support to Posternak's contention that phosphorus is loosely combined with carbohydrates in corn; or it may be that freezing caused the breakdown or altered the permeability of cell walls so that enzymes were permitted to come in contact with some amylo-phosphoric-acid complexes and split-off acids.

More nitrogen was obtained from hard than from soft Illinois corn starches. Smaller amounts were found in foreign starches. Since the amount of nitrogen is, no doubt, influenced by impurities in starch, these results are of questionable value.

Titratable acidity was higher in the harder samples while pH values were correspondingly lower. Since fatty acids are monobasic, they cannot, when esterified, contribute to the acidity of corn starch. In all preparations, however, there may be some free fatty acids. Possibly Lehrman's "adsorption complex" may help account for acidity. Phosphoric acid, on the other hand, is tribasic and so one or two of its acid hydrogens may be free to contribute to acidity.

TABLE 1

Characteristics of Starch From Several Varieties of Corn

Starch No.	Corn variety	Type of starch predominating	Method of propagation	H_2O	Ash	N	P	pH	Titratable acidity	Fatty acids
				pct.	*pct.*	*pct.*	*pct.*			*pct.*
9	Golden Beauty	Hard	Open pollinated	10.9	.10	.55	.044	5.4	3.0	1.50
10	Sutton	Hard	Open pollinated	11.7	.08	.21	.036	5.9	1.9	0.45
11	Sutton [1]	Hard	Open pollinated	13.6	.06	.18	.017	5.5	1.9	0.44
12	Pride of Saline	Hard	Open pollinated	11.3	.12	.48	.024	5.2	2.7	0.52
13	h. h.	Soft	Sib-crossed	12.4	.05	.18	.019	5.6	1.5	0.39
14	Argentina	Hard	Open pollinated	12.4	.04	.16	.020	5.9	1.1	0.62
15	African	Soft	Open pollinated	9.4	.06	.14	.009	6.0	1.3	0.44

[1] Frozen on the cob for one week.

What is responsible for acidity of corn starch appears still to be unknown.

SWELLING POWER, VISCOSITY, AND ALKALI-LABILE PROPERTY

Swelling power of starch granules and viscosity of starch suspensions are properties which determine to a large extent the consistency of starch-water mixtures.

Swelling power of starch is defined by Ripperton (1931) as "the volume in cubic centimeters of swollen granules of 1 gram of air-dried starch obtained by using the standard procedure." Swelling power, according to Alsberg (1926), depends upon the following factors: inherent swelling power of a given variety, relation of mass of swelling substance to surface area, and ease with which the anatomical structure may be softened by heat. Ripperton adds environment as another factor. Starch granules that have been ruptured either by chemicals or by grinding do not swell.

Viscosity, according to Rask and Alsberg, is "the resistance due to internal friction which viscous substances offer to deforming forces." A substance is viscous which will deform permanently to a degree directly proportional to the deforming force applied.

Ostwald (1913) listed at least 10 factors that influence viscosity; experimentation and theorization concerning viscosity of starch suspensions have occupied many investigators.

Cereal starches, such as corn, rice, and wheat, tend to produce suspensions of low viscosity at relatively high concentrations; while non-cereal starches—potato, tapioca, and arrowroot—form highly viscous suspensions at relatively low concentrations. Size, which once was thought to influence viscosity, has been found, as such, to have little effect. Ripperton found that differences in viscosity occurred naturally within a given species. Some potato suspensions were very viscous while others were not. He noted that weather, soil conditions, and length of storage period affected viscosity within a single strain, and related the differences observed to the degree of hydration and cation content of the amylophosphoric acid esters. Wolff (1927) reported that viscosity decreased with release of cations from starch.

Work by Maquenne and Roux (1906) indicated that the insoluble portion of starch granules were paste forming, a view accepted and substantiated by others. According to Harrison (1911) anything that breaks up swollen granules reduces viscosity.

In spite of the volume of work on viscosity measurements no standard method for its determination and no viscometer which suits every investigator have been perfected. Results are, therefore, not easily reproducible.

Native starch contains practically no reducing compounds, but treatments accorded it in manufacture and preparation for tests undoubtedly produce some materials which are quickly attacked by hot aqueous alkali. These materials, Taylor, Fletcher, and Adams (1935) have designated "alkali-labile." Natural characteristics of a particular starch may influence the amount of this material formed during processing and thus aid in differentiating starches. Alkali-labile values are empirical. Taylor states that with care, they can be duplicated on like samples.

Experimental Procedure

Swelling-power determinations were made according to the methods of Ripperton (1931). Amounts of corn starch equivalent to one-tenth of a gram of dry starch were mixed with 10-ml. portions of cold, boiled, distilled water in test tubes and heated for 15 minutes in boiling water. The mixtures were poured into 25-ml. graduate cylinders; the test tubes were rinsed with boiled water; the rinse waters were added to the cylinders, the cylinders filled to the mark, and set in a cool place over night. Ten times the number of milliliters occupied by the swollen starch represents "swelling power."

Relative viscosity of one-per cent samples of each corn starch was obtained by using a modification of the procedure of Taylor and Beckman (1929). One-half gram of moisture-free corn starch was heated with 10 ml. of cooled, boiled, distilled water for five minutes after suspension reached 97°C.(206.6°F.). The suspension was washed into a 50-ml. volumetric flask and made up to volume with boiled water. The flask was immediately immersed in a water bath, kept at 35°C.(95°F.). The suspension was put in an Ostwald viscometer and the time required for the solution to pass from the constriction at the top of the measuring bulb to that at the bottom was measured. This was compared with the time of flow of water through the same distance. Since the solutions in this investigation did settle, contrary to Taylor and Beckman's (1929) findings, three readings were taken, the solutions shaken, three more taken, and so on until 12 readings were made. The average of these 12 readings divided by the time required by water is expressed as "relative viscosity."

Alkali-labile determinations were made according to the methods of Taylor, Fletcher, and Adams (1935).

Discussion of Results

Four determinations of swelling power, under conditions as nearly identical as possible, gave results which showed no appreciable dif-

ferences between starches, except that soft starch, No. 13, with largest granules did exhibit a slightly higher swelling power.

That size of granules within a given species of starch may influence viscosity receives some support in this investigation. The softest Illinois starch, with largest granules and with nitrogen content the same as two other varieties, was less viscous than the others. The granules of treated and untreated Sutton corn starch and the foreign starches showed about the same range of sizes when examined with the microscope. The untreated native corn which showed a greater proportion of the smaller granules was the most viscous of the group. In accordance with the observations made on the soft and medium hard starches, the two hard native starches should have been most viscous. Both, however, were less viscous than some others, but they also had more nitrogen associated with them which might have been responsible for reducing viscosity.

Results from this investigation showed no correlation between swelling power and relative viscosity. They suggest, however, a possibility that viscosity may decrease as swelling power increases. There was also no evidence of a quantitative relationship between viscosity and phosphorus content. This might be interpreted as further evidence that phosphorus is distributed quite irregularly throughout the starch granule. That fatty acids contribute to viscosity was given some support in that starches, exclusive of Sample 9, associated with the larger amounts of fatty acids were the most viscous ones. The differences in amounts of fatty acids, however, may be within "experimental error" and it is suggested that kind of fatty acids associated with starches, rather than amount, may be the more important factor.

Ripperton noticed that starches of greatest viscosity were unsaturated as to cations and had a low pH. As the present starches were not only prepared in the same way but five were grown under similar conditions, it seems probable that the natural cations of these five were identical and that any ion exchange which occurred during preparation was the same in character if not in degree. A cation analysis of these starches might reveal some helpful information, especially as the harder starches appear to contain the most ash. The hardest Illinois starches, 9 and 12, were most acid. Freezing corn during early maturity decreased viscosity and pH (Sample 11). No correlation was apparent between pH and viscosity.

More alpha amylose (insoluble fraction) was obtained from the hardest, Sample 12, than from any other Illinois corn starch. If one may believe that nitrogenous impurities were responsible for the low viscosities of Illinois hard starches, it may be interpreted to

mean that as the amount of alpha-amylose is increased, so too is the viscosity increased. Owing to incomplete rupturing of granules of soft starch no alpha-amylose figures for them are recorded (Table 2). Since the hardest starches contained most alpha-amylose, one might expect the softer starches to contain less and as a result form less viscous pastes. A comparison of relative viscosity of corn starch suspensions with some properties reported to influence viscosity is presented (Table 2).

Alkali-labile values of the different Illinois corn starches were so similar that they did not help distinguish the types. Differences between individual determinations of each starch were greater than those between the averages of determinations for each starch. The

TABLE 2

Comparison of Relative Viscosity With Some Properties of Corn Starch

Starch No.	Relative viscosity	Swelling power	P	Fatty acids	pH	Alpha-amylose
			pct.	*pct.*		*pct.*
9	2.24	39.5	.044	1.50	5.4	7.43
10	2.54	39.5	.036	0.45	5.9	6.85
11	2.42	40.7	.017	0.44	5.5	.....
12	2.36	40.2	.024	0.52	5.2	9.20
13	2.28	45.0	.019	0.39	5.6	.....
14	2.45	43.0	.016	0.62	5.9	10.70
15	2.26	39.0	.029	0.44	6.0	.....

averages were slightly lower than those reported by Taylor and Keresztesy (1936) for untreated starch. This is to be expected since corn starch used in this investigation did not receive the acid pretreatment which the commercial starch used by Taylor and Keresztesy had received.

SEPARATION AND CHARACTERIZATION OF THE AMYLOSES

The two substances said to make up starch granules have, so far, eluded definition. Every method used to separate them has resulted in a soluble and insoluble fraction. The quantity of each fraction in a given starch appears to be a function of the particular method used to obtain it. Various names have been given these components, among them alpha-amylose for the insoluble and beta-amylose for the soluble fraction. These terms are used in this investigation.

Taylor and Iddles (1926) observed, as did Alsberg, that unless granules were entirely disrupted separation was not complete. Rupturing may be accomplished by suspending starch in water and heating under pressure, by chemical reagents, and by grinding. All starches do not respond equally well to all methods and it is doubtful

if complete disintegration can ever be accomplished by heating. Commercial corn starch has been, apparently, successfully disrupted by grinding. The advantage of grinding is that the use of chemical reagents, which have been employed to cause some hydrolysis of corn starch, is avoided. A disadvantage, however, of grinding is that silica from the ball mill contaminates starch, a fact which does not seem to have been given much consideration by previous investigators.

For actual separation of amyloses following rupturing of granules, electrophoretic means were usually employed. Alpha-amylose migrated toward the anode while beta-amylose remained dissolved in the liquid above. Soluble salts associated with starch passed through the semipermeable membranes and thus purified the starch fractions in the central chamber of the three-compartment electrodialyzer. After electrodialysis Taylor and Iddles (1926) obtained from 11.5 to 15.6 per cent corn alpha-amylose from six determinations on commercial corn starch.

Taylor and Keresztesy's (1936) work on fractionation showed beta-amyloses to be definitely heterogeneous. Taylor and Morris (1935) found retrograded beta-amylose in the insoluble portion and devised a method for separating them. Values from 8.1 to 15.9 per cent alpha-amylose were reported by Taylor and his associates in the various papers. While there is remarkable agreement in these results compared with other published values, they represent such a range that any possibility of determining from them a true ratio of the amyloses in corn starch, provided such a ratio exists, is precluded.

Experimental Procedure

Grinding: Two hundred grams of each type of corn starch and 1,500 grams of quartz balls were ground in a four-gallon ball mill jar about 624 hours.

Electrodialysis: Two cells similar to that of Taylor and Iddles (1926, p. 715) were constructed. Each consisted of three compartments—cathode, anode, and the central one containing the material to be separated. duPont's No. 600 cellophane was used for membranes between the compartments. The electrodes were of platinum. These were connected to the 110-volt line with the positive electrodes at the bottom of the electrophoretic cell system.

Taylor and Keresztesy's method of separation formed the basis of the major portion of this work. Fifty grams of ground starch mixed with 100 ml. of methanol were added to 1,500 ml. of boiling distilled water and permitted to boil a few minutes before being cooled and introduced into the electrophoretic cell. Distilled water

was placed in the electrode compartments. Toluene was added and the material left until separation was effected. The upper portion was siphoned off and concentrated on an electric plate. The pasty precipitate was heated to boiling with about a liter of water, cooled, and returned with more water to the cell. This process was repeated four or five times. To the liquid portions was added methyl alcohol to precipitate beta-amylose. It was separated from the alcohol by filtration through a sintered glass funnel and dried, if necessary, on the electric plate. The alpha portion was centrifuged to separate it from adhering liquid, flocculated in methanol, centrifuged again, and dried in a tared beaker in an oven at 70°C.(158°F.).

The purity of this alpha-amylose was tested by Taylor and Morris' precipitation method. A three-per cent suspension was brought to the boiling point, cooled to room temperature, and 12 per cent aqueous sodium hydroxide solution was added until 2.5 per cent was present. After stirring a short time the clear solution was neutralized and then slightly acidified with cold dilute hydrochloric acid. (This concentration of amylose and alkali seemed to Taylor and Morris to be optimum for this purpose.) The precipitate of alpha-amylose was separated and washed repeatedly by decantation in a centrifuge, then flocculated with methyl alcohol, and the residue was dried to constant weight at 70°C.

Discussion of Results

Grinding for 624 hours in a ball mill, while sufficient to disintegrate commercial and hard starches, is not enough for soft starches, a fact not recorded heretofore. Unfortunately hard starches were examined first, and the difficulty encountered where soft starches were used was not anticipated or discovered in time to reorganize the technics. Thus, "alpha-amylose" discussed in this investigation refers to the purified insoluble product of electrodialysis of corn starch ground 624 hours and purified according to Taylor and Morris' method.

The four hard starches were contaminated with from .25 to 1.7 per cent of ball-mill material during grinding. This material, if present as a hydrogel, must influence viscosity and solubility relations. It may be that it and some entrapped carbohydrate material was the alpha-amylose that Taylor observed after grinding. Analysis of ash of both fractions indicated that ash went almost entirely with the alpha portion of hard starches. Soft starches were not appreciably contaminated. When the starch-methanol pastes were mixed with boiling water they formed apparently homogeneous mixtures of different degrees of thickness; the hard starches formed

thin suspensions, and the soft starches formed mixtures resembling cooked laundry starch.

Early in this investigation 100-ml. suspensions of ground starch were separated in a horizontal A. H. Thomas electrodialytic cell, using a direct current of 110 volts. Suspensions of commercial starches and No. 10 separated into water-clear liquids and pasty precipitates in two or three days. Four days were required when No. 10 was put in the large cell. This starch and No. 12, another hard one, were the only ones which separated so completely. Opalescence of varying degrees was noted when other starches were electrodialyzed. Silica hydrogels may explain part of the opalescence.

TABLE 3

Fractionation of Ground Starch in Electrodialysis

Starch		Alpha-amylose		Beta-amylose fraction	Amyloses (pure starch basis)	
No.	Amount	By electrodialysis [1]	By precipitation [2]		Alpha	Beta
	gm.	*gm.*	*gm.*	*gm.*	*pct.*	*pct.*
9	50	5.23	5.55	39.50	7.43	92.57
10	50	7.33	5.86	38.03	6.85	93.15
11	50	17.31	7.85	33.60	17.30	82.70
12	50	6.71	4.15	36.07	9.20	90.80
13	50	.....	7.87	.....	17.85	82.15
14	50	7.10	4.74	36.80	10.50	80.50
15	50	11.77	9.62	20.45	21.00	79.00

[1] According to Taylor and Keresztesy. [2] Preceding precipitate treated for retrograded amylose, according to Taylor and Morris.

After a few days of electrodialysis the solid portion of the hard starches filled so small a space in the cell that the liquid containing beta-amylose could easily be siphoned away. Soft-starch pastes, however, so completely filled the space that separation was effected only after centrifugation. Two-per cent suspensions of soft starch were much more gelatinous than five-per cent suspensions of hard starch.

The alpha-amylose portions from hard starches 9, 10, 12, and 14 in the electrophoretic cell were tan and pasty. They dried to a dark horny material, difficult to powder. Less than 11 per cent of the original ground starch was represented by the material recovered after purification by Taylor and Morris' method. Complete recovery of beta-amylose by precipitation was never accomplished. The liquid portion from the soft starches was so contaminated with alpha-amylose that quantitative measurements were valueless.

A rather complete separation of hard-starch alpha-amyloses appeared to be accomplished. The amount of alpha-amylose which may

have been responsible in part, for opalescence represented a very small fraction. More significant were the mechanical losses occurring during manipulation. Of the three Illinois hard starches most "pure"[1] alpha-amylose was separated from the hardest, No. 12; while the smallest amount came from No. 10, a medium hard variety, which accompanies larger amounts of alpha-amylose.

Neither amylose obtained in this study had all the characteristics described by Taylor. The alpha portion in its purest form contained much less fatty acid than did his, and the beta-amylose contained some fatty acids. No other reference to fatty acids in beta-amylose is known, nor is it known that those found were not due to experimental error. This seems unlikely, considering the uniformity of results. There seems to be no reason why fatty acids, as well as phosphoric acid, could not be esterified on carbohydrate residues of different sizes and consequently appear in both fractions.

Alpha-amylose, as obtained in the laboratory, has had so many opportunities to have its chemical and physical characteristics altered and to be contaminated with organic and inorganic substances that properties ascribed to it may not be those of the original starch fraction occurring in plants.

The results obtained in this investigation seem to support those who wish to disregard alpha- and beta-amyloses as entities. They support the possibility that (1) different particle sizes rather than materials may be responsible for the soluble and insoluble fractions; and (2) that the nonuniformity of results obtained from "identical" samples may be caused, in part, by differences in amounts and kinds of non-carbohydrate materials esterified to the individual particles of the aggregates making up starch granules.

SUMMARY

These results indicate that differences exist between hard and soft corn starches. Granules of hard starch appear to be slightly smaller and less rounded than soft granules. More non-carbohydrate material was found associated with hard corn starch. Measurements of titratable acidity and pH values are probably of little value in classifying corn starches.

Hard starches appear to form more viscous pastes than do soft ones of approximately the same granule size.

Grinding corn 624 hours, while sufficient to disintegrate hard starches, does not completely disrupt soft starches. Portions of

[1] "Pure" starch was made by deducting moisture and ball-mill ash from sample weights.

alpha-amylose separated by electrodialysis from soft starches were accordingly contaminated with whole granules.

Grinding was responsible for ash contamination of hard starches. Soft starches were not appreciably contaminated.

Electrodialysis at 110 volts was not in all cases sufficient to separate five-per cent suspensions of ground starch into a clear solution above a gelatinous or pasty precipitate of alpha-amylose. Consequently beta-amylose obtained from alcohol precipitation of opalescent liquid was contaminated with a trace of alpha-amylose. Electrodialysis at higher voltages may be advantageous.

Sufficient evidence to prove that either amylose exists as an entity or group of entities is not apparent. So many opportunities occur for contamination and chemical and physical change that "alpha-amylose" could be any one of a variety of substances.

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COMPARISON OF ORGANOLEPTIC AND PHYSICOCHEMICAL METHODS FOR DETERMINING QUALITY IN FRESH, FROZEN, AND CANNED LIMA BEANS[1]

AMIHUD KRAMER AND C. H. MAHONEY
Department of Horticulture, University of Maryland, College Park, Maryland

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The primary object of this investigation was to measure, by rapid physicochemical methods, the quality of frozen and canned lima beans. It is generally conceded by food technologists and lima-bean canners that, when used by experts, organoleptic tests are satisfactory in evaluating the quality of canned or even frozen samples. However, the personal element is a strong factor in such organoleptic tests, and even though a large number of samples are graded, biochemical workers contend that a completely unbiased report is not possible. Therefore, if it could be shown that either physical or rapid chemical methods would accurately measure the quality of frozen and canned samples whose quality had been varied by previous treatment of the raw product, then they should be acceptable to food workers provided they compared favorably with commonly accepted methods of measuring quality.

Diehl, Dingle, and Berry (1933), Kohman and Sanborn (1934), and others have shown that enzyme activity is largely responsible for off-flavors developing in unblanched, frozen vegetables. It would appear then that enzyme activity might be one measure of quality. Tressler and Evers (1936) have given detailed procedures for the measurement of catalase activity for this purpose. Such a method, however, cannot be used to measure quality since Kertesz, Dearborn, and Mack (1936), Arighi, Joslyn, and Marsh (1936), Tressler, Mack, Jenkins, and King (1937), and Tressler (1938) have definitely shown that catalase as well as other thermolabile enzymes can be inactivated by a relatively short blanch. Smart and Brunstetter (1936), on the other hand, found catalase activity in lima beans after a nine-minute blanch at 88°C.(190°F.) which is not in accordance with results of other workers.

Tressler (1938) and Fitzgerald (1938) have suggested that vitamin C might be a good indicator of quality since both quality and

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vitamin C content are influenced in the same direction by factors such as variety, overmaturity, fertility level, interval between harvesting and blanching, slow cooling after blanching, and overcooking influence. The exact correlation between vitamin C and quality as modified by the above factors has not been definitely shown.

EXPERIMENTAL PROCEDURE

Materials: Lima-bean varieties were grown in Maryland at Ridgely on the eastern shore, and at College Park. Henderson and Early Baby Potato varieties grown at Ridgely were harvested with a commercial digger and threshed in a large commercial viner within 45 minutes of harvest. Eleven other varieties (Table 5) were grown at College Park where the plants were pulled, pods removed, and shelled in a Junior pea and lima bean huller.

Immediately after vining, the baby-lima varieties were graded, the No. 2 sieve beans ($\frac{7}{16}$ to $\frac{9}{16}$ of an inch) were removed, washed in cold water, and the white beans were discarded. The prevailing sieve size of the Potato limas was handled in a comparable manner.

Blanching, Canning, and Freezing: As it is definitely known that holding beans for any length of time after vining seriously affects quality, preblanching [2] treatments of 2, 6, 10, and 22 hours were selected for the 1938 Ridgely material. Lots of these samples were then blanched in water at 91°C.(196°F.) for periods of 45, 90, 180, and 300 seconds, immediately cooled in cold running water, and then put into No. 1 cans. Half of the samples to be frozen were drained and placed in cans; the other half of the samples to be frozen and all samples to be cooked were covered with two-per cent salt brine. All cans were sealed with a semiautomatic closing machine. Six cans of each treatment were frozen by direct contact with dry ice in an insulated box and were then placed in storage at —17°C.(1.4°F.). Twelve cans of each treatment were processed in a retort at 121°C.(250°F.) for 20 minutes. Samples of Early Baby Potato as well as Henderson of the 1939 Ridgely tests were given preblanching treatments of two and six hours, were separated in 13-per cent brine, and then were blanched for 30 and 60 seconds at 99°C.(210°F.).

The 13 varieties grown at College Park in 1939 were harvested during a warm spell in late August. The samples to be frozen were given a uniform blanch of one minute and the samples to be canned were blanched for three minutes at 99°C.(210°F.). The methods used for freezing and canning were similar to those used at Ridgely.

[2] Preblanching, as used here, refers to the time interval between harvest and blanching.

Organoleptic Tests: The frozen samples were thawed by placing the material in boiling water and allowing them to cook for 14 minutes after the start of the second boiling.[3] The cooking time from the second boil was later increased to 17 minutes since it was found that 14 minutes were not sufficient to completely cook the samples.

The frozen samples were cooked and then graded on a score-card basis for odor, color, texture, and flavor by four groups of judges averaging six men per group. The scores of poor, fair, and good were given numerical scores of 1, 3, and 5, respectively, so that the data could be statistically analyzed. The final score given each sample was the average given by the four groups of judges for the four items scored. The canned samples were graded independently by four groups of judges.

Enzyme Tests: Peroxidase activity was determined by the guaiacum colorimetric method suggested by Bedford (1938) modified to the extent that it was placed on a semi-quantitative basis by measureing the color intensity with a colorimeter.

Catalase was determined qualitatively on a spot plate by using the reaction described by Schönn (1870).

Pigment Tests: Methanol-petroleum ether pigment extracts were also made and measured with a colorimeter.

Iodine Test: The iodine-threshold value was obtained by a modification of the method suggested by Joslyn, Bedford, and Marsh (1938) for the measurement of vitamin C. Ten-gram samples of beans were ground with two grams of quartz sand in the presence of 25 milliliters of a mixture of two per cent metaphosphoric and eight per cent sulphuric acid. The acid extract was then strained through cheesecloth of double thickness and made up to 60 milliliters with the addition of eight per cent sulphuric acid. Two milliliters of a one-per cent starch solution were added, the solution stirred, and an aliquot of 10 milliliters removed and placed into one of the two tubes of a Bausch and Lomb quartz colorimeter. A definite amount of .01 N iodine solution was then added to the rest of the sample.[4] Five drops of a .04-per cent aqueous methylene blue solution were added to the 10-milliliter aliquot which served as standard. The tube containing the standard was raised to read 15 on the colorimeter. An aliquot of the solution to which iodine had been added was then placed in the other tube of the colorimeter and adjusted to the color of the standard. The value thus obtained was designated as the iodine-threshold value.

[3] As suggested by Mrs. Boggs in personal correspondence.

[4] The amounts added were three milliliters to the frozen samples, 1.2 to the canned samples, and five to seven milliliters to fresh samples.

The advantages of this method are its rapidity and simplicity. A reading can be made in five minutes, and can be made in the field or canning plant as well as in the laboratory. The iodine reaction is much more stable than the one obtained with the dye dichlorophenol

TABLE 1

Organoleptic and Iodine-Threshold Values of Henderson Lima Beans Frozen Dry and in Brine, as Affected by Preblanching and Blanching Periods

Treatments		Tests				
		Organoleptic		Iodine-threshold		
				Brine frozen		Dry frozen
Preblanch	Blanch at 91°C. (196°F.)	Brine frozen	Dry frozen	Beans	Brine	
hr.	*sec.*					
0- 2	45	4.5	4.3	12.6	5.1	19.1
	90	4.5	4.3	11.9	11.6	14.7
	180	4.0	4.0	10.4	4.3	14.0
	300	4.0	3.7	9.4	4.6	14.0
5- 6	45	3.8	3.8	11.6	10.6	17.6
	90	3.8	3.5	9.9	5.2	13.1
	180	4.2	3.8	9.9	4.8	12.9
	300	3.5	3.8	8.7	4.6	12.2
9-10	45	3.5	.....	11.2	15.1	12.8
	90	3.3	.....	10.0	6.2	12.2
	180	3.8	.....	10.3	7.1	9.8
	300	3.0	.....	8.1	5.7	10.8
21-22	45	2.2	1.3	8.6	5.4	11.3
	90	1.3	1.7	8.3	5.1	10.2
	180	2.5	1.7	7.6	4.5	9.2
	300	1.3	1.8	8.3	4.2	8.1
Difference required for significance.....		0.83	0.55	0.90	.....	2.00
"F" values for						
Preblanching.....		184.2[1]	182.4[1]	69.5[1]	0.6	50.7[1]
Blanching.....		1.4	0.3	45.7[1]	0.9	26.6[1]
Replicates.....		9.6[1]	5.5[1]	2.2	.....	.....

[1] Significant beyond the one-per cent point.

indophenol, which is in general use for vitamin C determination. By the use of this method only the difference between treatments is measured, whereas by the titration method a measure of the total iodine reducing substances is obtained.

Since Miller and Robbins (1937), Guthrie (1937), and others have shown that iodine reduces not only ascorbic acid but also glutathione and other materials, it is inaccurate to call this procedure a

method for determining vitamin C. Therefore it is not valid to convert iodine-threshold values into milligrams of ascorbic acid. For the purpose of quality determinations on frozen or canned samples, however, such a nonspecific reaction involving several substances

TABLE 2

Organoleptic and Iodine-Threshold Values of Canned Henderson Lima Beans as Affected by Preblanching and Blanching Periods

Treatments		Tests		
Preblanch	Blanch at 91°C. (196°F.)	Organoleptic	Iodine-threshold	
			Beans	Brine
hr.	*sec.*			
0- 2	45	3.5	25.0	80.0
	90	3.8	22.0	43.3
	180	3.8	22.1	43.0
	300	3.5	16.6	25.5
5- 6	45	2.9	18.1	70.0
	90	3.1	18.0	42.6
	180	2.5	17.3	25.0
	300	2.4	15.0	19.9
9-10	45	3.5	20.2	60.0
	90	3.6	17.5	34.1
	180	2.2	16.8	26.2
	300	3.2	15.5	12.4
21-22	45	2.0	13.7	11.9
	90	2.5	14.9	13.4
	180	1.7	14.6	14.1
	300	2.2	12.7	11.5
Difference required for significance		0.80	3.28	16.68
"F" values for				
Preblanching		22.9[1]	25.9[1]	6.3[1]
Blanching		4.7[1]	12.1[1]	7.4[1]
Replicates		64.9[1]	1.4	.....

[1] Significant beyond the one-per cent point.

might possibly show a closer correlation with quality than a single specific test.

RESULTS AND DISCUSSION

The 1938 comparisons of the organoleptic and iodine tests are given for the frozen samples (Table 1) and for the canned samples (Table 2). These data were analyzed by the variance method and the highly significant "F" values clearly indicate that the preblanch

(interval between harvest and blanching) was the most important factor affecting the quality of these samples. The influence of the preblanch treatments on the quality of the frozen samples was more severe than on the canned samples as measured both organoleptically and by the iodine method. The significance of the preblanch treatments was essentially the same on the canned samples as determined by both methods, but on the frozen samples the

TABLE 3

Organoleptic and Iodine-Threshold Values of Frozen Lima Beans as Affected by Preblanching, Blanching, Varieties, and Brine Separation

Variety and preblanch	13% Brine separation	Blanch at 99°C. (210°F.)	Organoleptic	Iodine-threshold
Early Baby Potato, 2 hours	Floated	30	3.5	10.02
		60	4.4	7.77
	Sank	30	3.6	10.53
		60	3.4	7.80
Early Baby Potato, 6 hours	Floated	30	4.1	8.19
		60	3.7	7.41
	Sank	30	3.3	8.61
		60	3.7	7.38
Henderson, 2 hours	Floated	30	3.3	8.28
		60	2.8	7.59
	Sank	30	2.7	8.22
		60	2.4	7.59
"F" values for				
Preblanching			0.19	130.19 [1]
Blanching			0.99	307.57 [1]
Varieties			18.30 [1]	14.77 [1]
Brine separation			4.55 [2]	5.88 [2]
Replicates			5.97 [1]	2.23

[1] Significant beyond the one-per cent point. [2] Significant beyond the five-per cent point.

organoleptic tests were more sensitive in detecting these quality changes. The iodine test, however, was slightly more sensitive in detecting changes between the two- and six-hour preblanch in 1938. The two preblanch treatments used in 1939 (Table 3) were two and six hours.

It is evident from the data (Table 3) that the quality changes owing to preblanch were not detectable by the organoleptic test but these differences were highly significant as measured by the iodine test. This essentially confirms the 1938 results where the organoleptic tests were not quite significant in measuring quality changes

between the two- and six-hour preblanch treatments. It should be pointed out that the iodine values of the frozen and canned samples cannot be compared directly since three and 1.2 milliliters of iodine, respectively, were added to these samples. These values could be converted into milligrams of ascorbic acid, but since only a portion of the total iodine reducible substances can be assumed to be ascorbic acid, such a conversion would not be valid.

There was no detectable difference in quality of beans frozen dry as compared with those frozen in brine when placed in hermetically sealed cans. Therefore, the brine pack was not used for the 1939 material.

TABLE 4

Correlations Between Organoleptic and Iodine-Threshold Values of Frozen and Canned Lima Beans

Treatment	Correlation coefficient
Beans, frozen in brine	.83
Beans, frozen dry	.79
Blanching constant at 45 seconds	.90
Blanching constant at 90 seconds	.87
Beans, canned	.75
Brine,[1] canned	.58

[1] Iodine-threshold determination of the brine, and organoleptic test of the beans of the canned samples. Standard deviation is .04 or less.

The "F" values (Tables 1 and 2) clearly show that the blanching treatments did not affect quality as significantly as the preblanching treatments. In fact the differences are not significant for the frozen material when measured by the organoleptic tests. These "F" values are significant for both organoleptic and iodine tests on the canned material and for the iodine test on the frozen samples. The latter is apparently more sensitive to changes owing to blanching than is the organoleptic test. The "F" values presented (Table 3) show that blanching affected quality more significantly in 1939 than preblanching, but, as has already been pointed out, only two preblanching treatments were used on this material.

The minimum blanching period for 1939 was reduced to 30 seconds, as the 45-second blanch used in 1938 inhibited catalase and peroxidase activity. Consequently in 1939 two out of 18 samples blanched for 30 seconds showed slight evidence of peroxidase activity. This would seem to indicate that a 30-second blanch at 99°C. 210°F.) for material in hermetically sealed cans was very close to the critical point for enzyme activity.

The degree of relationship between the organoleptic and iodine tests as measures of quality has been determined by the use of correlation coefficients. These values are presented (Table 4) and in all cases but one the coefficients are high. The correlation coefficient between these two tests for beans frozen dry is .90 when the blanching time has been held constant for the variable preblanch treatments. Therefore it would appear that the iodine test would be quite suitable as a measure of quality of frozen lima beans, provided the beans were not overblanched. Overblanching affects the iodine-reducible substances to a greater degree than the quality as measured by commonly accepted organoleptic tests. Even though the correlation coefficient of .75 for the canned samples is not as large as for the frozen samples, yet it is high enough to show that there is a definite relationship between these two tests on canned material. Furthermore, the correlation coefficient of .58 between the iodine test on the brine of the canned samples with the quality of the beans measured by organoleptic tests would indicate that the iodine-reducible substances have been cooked out of the beans in processing. If this were taken into account the correlation then would probably be almost as high as that of the dry frozen samples.

The results of a single year on 13 varieties (Table 5) show that in general there is a fairly good agreement between the organoleptic and iodine tests. These data are presented simply to show that bean varieties vary in quality. In fact the variations in iodine value of the fresh samples were so great that a constant quantity of iodine could not be used for all samples. Consequently they were converted to milligrams of ascorbic acid so that a comparison could be made. It is quite evident that Henderson and Illinois Large Podded are the least desirable from the quality standpoint in their respective groups, but it is not possible because of the limited number of samples of each variety to determine as yet the mathematical relationship between the two tests as influenced by variety. There is no apparent relationship between the iodine test on the fresh material and either the iodine or organoleptic tests on the frozen samples as determined by the rather meager data of 1939.

Practical Application: As there is good agreement between the two tests as measures of quality variations, owing to preblanching periods and varieties, it would appear then that the iodine test might be tried by canners and freezers of lima beans to measure quality. The very simple procedure is as follows: grind a 10-gram sample of frosted lima beans in an acid mixture (approximately 50 c.c. of two per cent metaphosphoric, and eight per cent sulphuric), add a little (one or two c.c.) one-per cent soluble starch solution and 3.5 c.c.

of .01 N iodine. A faint color or no color would indicate high quality. Increasing color intensity would indicate decreasing quality. Canned material may be tested in a similar manner, except that only 1.5 c.c. of the iodine solution should be added. The quantities of the acid and starch used need not be measured accurately. The iodine, however, should be measured carefully, and made up fresh every two or three days.

TABLE 5

Organoleptic and Iodine-Threshold Values on Fresh and Frozen Samples of 13 Varieties of Lima Beans

Varieties	Fresh—iodine-threshold [1]	Frozen	
		Iodine-threshold	Organoleptic
Baby types			
Henderson	38	7.73	2.4
Baby Fordhook	45	9.40	3.3
Early Baby Potato	49	9.57	3.0
Regular Baby Potato	43	9.90	3.8
Maryland Thick Seeded	49	9.83	3.7
Potato types			
Fordhook	42	12.97	4.9
Concentrated Fordhook	43	12.80	4.2
Early Giant	45	13.77	4.2
Improved Giant	44	12.17	3.8
Early Giant Podded	45	14.27	3.5
Early Wilson	42	13.40	3.6
Dreer's Wonder Bush	36	12.97	3.5
Illinois Large Podded	41	8.97	2.1
Difference required for significance	....	1.27	1.21
"F" values for			
Varieties	....	14.99 [2]	8.62 [2]
Replicates	....	0.26	10.76 [2]

[1] Calculated as milligrams of ascorbic acid in 100 grams. [2] Significant beyond the one-per cent point.

SUMMARY

Organoleptic and physical chemical quality tests were made on fresh, frozen, and canned lima beans. Differences in quality were induced by varying the preblanching and blanching periods of two of the 13 varieties used, and by brine separation. Qualitative determinations of catalase activity, and semiquantitative determinations of peroxidase activity and pigment content were apparently unsuitable as quality indices for frozen and canned lima beans.

The iodine-threshold test appeared to be a good measure of quality, since a correlation of .90 was obtained between the iodine and

organoleptic tests on material which underwent various preblanching treatments. Furthermore, of the factors affecting quality which were studied, preblanching was found to be the most important. There was also good agreement between the two tests in measuring the quality of different varieties. Overblanching, however, reduced the iodine value significantly more than the organoleptic value. There was an inverse relation between the two tests as affected by brine separation.

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THE HEME PIGMENTS OF CURED MEATS

I. PREPARATION OF NITRIC OXIDE HEMOGLOBIN AND STABILITY OF THE COMPOUND[1]

W. M. URBAIN AND L. B. JENSEN
Research Laboratories, Swift & Company, Chicago, Illinois

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Unlike fresh meats, the so-called cured meats retain their redness on cooking. It is inferred from this that the curing process changes the chemical nature of the meat pigment. Chiefly through the work of Haldane (1901) and Hoaglund (1914) this difference in reaction of the pigments toward heat has been shown to be due to the formation of nitric oxide derivatives of the meat pigment as a result of the chemical interaction of the pigment with the nitrite, either added directly in the curing mixture or obtained through microbial reduction of nitrate.

Since the work of Haldane and Hoaglund, the existence of a red pigment different from the blood pigment hemoglobin has been demonstrated in muscular tissue by Günther (1921), Kennedy and Whipple (1926), Hektoen, Robscheit-Robbins, and Whipple (1928), and Theorell (1932, 1934a, b, c, d). Haldane and Hoaglund assumed the pigment of meat to be identical with the blood pigment. Determination of amounts of the muscle hemoglobin or myo-hemoglobin, by Shenk, Hall, and King (1934) and by Watson (1935), occurring in the muscular tissue of several animals indicated that this pigment comprises nearly all of the tissue pigment. Since the slaughter of animals by modern methods includes a bleeding process which removes most of the blood, it is suggested that, in light of the isolation of the muscular pigment, the pigments of cured meats are not essentially nitric oxide hemoglobin and nitric oxide hemochromogen, as they have been called in the past, but instead are chiefly nitric oxide myo-hemoglobin and nitric oxide myo-hemochromogen.

While it is recognized that the true meat pigments are mainly derivatives of myo-hemoglobin, it is the purpose of this investigation to study in a systematic fashion the nitric oxide derivatives of hemoglobin itself, since it is easily possible to obtain hemoglobin in sufficiently large quantities; whereas, the difficulties of isolation of myo-hemoglobin are considerable. The close relationship of the two

[1] This investigation was carried out in the Research Laboratories of Swift & Company, Chicago. The Institute of American Meat Packers participated in a portion of this study through Mr. D. A. Greenwood, working under a grant to the institute from the Corn Industries Research Foundation.

pigments, hemoglobin and myo-hemoglobin, undoubtedly will permit correlation of the properties of hemoglobin derivatives with those of meat pigments. It is to be expected that in a study of the properties of the nitric oxide derivatives it will be necessary to make what are apparently digressions, that is, the properties of certain other compounds and the development of special techniques will have to be investigated in order to obtain a more complete knowledge of the nitric oxide derivatives. From this study of the fundamental chemistry of the pigments, it is hoped that a better understanding of the processes and factors involved in the development of good color in cured meats will be obtained.

THE CURING PROCESS

Depending upon the type of meat product involved, there are several types of procedures employed to cure meats. They may be described as follows: (1) Brine cure. The meat is soaked in a solution containing sodium chloride, possibly sugar, and sodium nitrate or sodium nitrite or both. The period of cure is governed by the size of the pieces of meat, sufficient time being allowed for complete diffusion of the curing agents into the center of the meat. At the end of the cure, the product may be smoked or otherwise processed. (2) Pump cure. In this type of cure the brine solution is pumped through the vascular system of the tissue, thus reducing enormously the time required for the complete diffusion of the curing agents. (3) Dry salt cure. A mixture of the dry curing materials is spread on the meat product, and it is stored for a period until diffusion into the meat has occurred. Smoking or other processing follows the cure. (4) Cure for comminuted meats. In the case of ground-meat products, such as sausage, the curing agents are incorporated by direct mixing. A tempering or curing period, after mixing, may take place and, following this, other processing.

All of these processes eventually lead to the formation of nitric oxide myo-hemoglobin, according to the reaction:

$$NO + \text{Myo-hemoglobin} = \text{Nitric Oxide Myo-hemoglobin.}$$

As mentioned above the source of the nitric oxide is nitrite or, more properly, nitrous acid, since the pH of meat is normally on the acid side, ca. 6. Nitric oxide myo-hemoglobin is then the first pigment of cured meats. By heat it is converted into nitric oxide myo-hemochromogen, a denatured protein. The fact that this hemochromogen compound is red is the basis for employing nitric oxide derivatives as cured meat pigments. The origin of this custom for cured meats is lost in antiquity.

Nitric oxide myo-hemoglobin is the basic and prime pigment of cured meats. In this paper is reported an investigation of some of the properties of the closely related compound, nitric oxide hemoglobin.

EXPERIMENTAL PROCEDURE

Preparation of Hemoglobin: Hog's blood was used as a source of hemoglobin. It was collected by severance of the jugular vein and either puncture or severance of the carotid artery. The blood was citrated immediately on collection, to prevent coagulation, and following this it was thoroughly oxygenated by gently shaking it in contact with air for about five minutes. The blood was then quickly chilled to slightly above 0°C.(32°F.).

The cells were removed from the serum by centrifugation at a temperature slightly above 0°C. The cells were washed six times with a volume of two per cent sodium chloride equivalent to the volume of serum removed, chilled to 0°C., the cells being separated from the wash sodium chloride solution by centrifugation each time.

The hemoglobin was released from the cells by dilution with chilled distilled water so that the final volume was roughly five times that of the original whole blood. This solution was next treated with about 10 per cent by volume of aluminum hydroxide cream, prepared according to the directions of Marshall and Welker (1913). This aluminum hydroxide was then centrifuged out along with the stroma and proteins other than oxyhemoglobin precipitated by the hydroxide.

This solution of oxyhemoglobin so obtained constituted the "stock" solution. It could be kept at 10°C.(50°F.) for about 10 days without the use of a preservative before decomposition set in. A Van Slyke analysis for oxygen capacity using the modification of Urbain and Greenwood (1940a) along with a spectrophotometric determination of the combined hemoglobin and methemoglobin concentrations, according to the method of Drabkin and Austin (1935), was made before this solution was used. The difference between the spectrophotometric result and the Van Slyke result was the amount of methemoglobin present. Unless this was zero, the solution was not used. Deterioration of the stock solution was always indicated by a lowering of the oxygen capacity as determined by the modified Van Slyke method.

Precautions were taken at all times to prevent contamination of solutions with bacteria, since bacteria are an important cause of changes in hemoglobin pigments, as was shown by Jensen and Urbain (1936). It is important to distinguish between the purely

chemical changes and those brought about by bacterial action in work on this compound and its derivatives. Data showing the bacterial counts obtained from typical stock solutions immediately after preparation (Table 1) indicate, in general, a very small bacteria count despite the impossibility of using aseptic technique in every step.

Preparation of Nitric Oxide Hemoglobin: Nitric oxide hemoglobin was prepared by action of nitric oxide on hemoglobin in the absence of air; the apparatus shown (Fig. 1) was used for this purpose. Essentially it consists of a chamber which can be evacuated and shaken simultaneously. *I* is a liter flask, supported by the rub-

TABLE 1

Bacterial Counts From Typical Stock Solutions Immediately After Preparation

Preparation	Bacteria per ml.[1]
83	7
87	20
88	11
91	7
95	1
104	0
130	0

[1] Counts obtained by incubating at 20°C.(68°F.) for 72 hours, using North Gelatin-Agar-Difco as a nutrient medium.

ber stoppers *F* and *F'*. These stoppers are set in the two square metal plates *G* and *G'*, which are held together by four bolts, one at each corner, as shown. The wheel *A* is connected by the rod *B* to the plate *G*, so that turning of the wheel produces a shaking motion. The whole apparatus is supported just below *F'* on a metal shaft perpendicular to the plane of the drawing; this is the center of the shaking motion. *C* is a glass fitting similar to that commonly used on the Van Slyke blood apparatus for admitting gases or liquids through a rubber-tipped capillary, employing mercury to seal the connection. *D* is a three-way vacuum stopcock. *J* and *N* are two-way vacuum stopcocks. *P* is a trap for the manometer *O*. *E* and *M* are pressure hoses connecting the two sections of the apparatus. A side tube *Q* permits the necessary glass blowing. *R* is a vacuum oil pump.

The following procedure was employed in making nitric oxide hemoglobin with the above apparatus: The pump was started; cocks *J* and *N* were closed and *D* turned so that *E* was connected. When the chamber was evacuated, the cup *C* was filled with a solution of

hemoglobin of the proper strength, and *D* turned so that the contents of *C* were sucked into the chamber. The cup *C* was kept filled by pouring more of the solution into it before air could be sucked in, and in this way the chamber was filled to the desired amount (usually about half full). Following the last portion of the solution, about three to five drops of capryl alcohol were admitted to the chamber to prevent excessive foaming of the solution during the subsequent operations. *D* was then turned to connect *E* and the shaking was started.

The combined evacuation and shaking quickly removed the dissolved gases from the solution as well as from the space above the liquid. In a comparatively short time the oxyhemoglobin was converted to reduced hemoglobin.

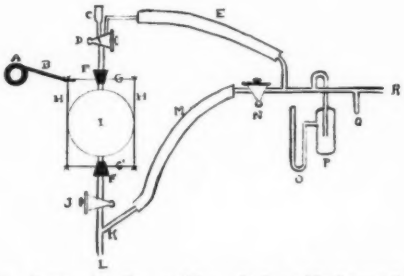

FIG. 1. Apparatus for making nitric oxide hemoglobin.

Nitric oxide was prepared by the action of nitric acid on copper and washed free from nitrogen dioxide by passing through water into gas-storage bottles similar to those described by Van Slyke and Hiller (1928), except that the necessity of using a Hempel pipette was eliminated by replacing the fitting for that pipette on the gas bottle with a rubber hose ending in a rubber-tipped capillary. By means of this capillary, connection could be made through the fitting *C* (Fig. 1) and the chamber *I* could be filled with nitric oxide. Stopcock *D* was then closed and the shaker run for three minutes, nitric oxide hemoglobin apparently being formed immediately. The excess nitric oxide was removed by combined evacuation and shaking.

The nitric oxide hemoglobin at this final stage was in the chamber *I* under vacuum. One of two alternative procedures was then used, depending upon the purpose for which the preparation was to be employed. If it was desired to study the reaction of the pigment in the absence of oxygen, a tube could be sealed onto the apparatus at *L*. To do this, *D* was closed, *N* opened, the pump *R* stopped, and *Q* broken open (to permit the necessary glass blowing).

The seal completed, *Q* was sealed shut and the system evacuated. *D* was then opened to *E*, followed by *J*. The nitric oxide hemoglobin flowed into the tube. If the reaction of the pigment with another substance was to be studied, this substance was placed in the sample tube before it was sealed on. In order to make the study quantitative, the tube was calibrated and thus a measured quantity of the solution of nitric oxide hemoglobin was admitted to the tube. When the tube was filled as desired, *J* and *D* were closed; the tube was then sealed off under vacuum. A fresh tube could be sealed on and the process repeated as often as required. In this way the reaction of the pigment in the absence of air with a number of materials could be studied from one preparation.

For studies in which it was desired to expose the preparation to the air, the solution was simply allowed to flow out of the chamber *I* through *L*, cocks *D* and *J* being open.

Spectrophotometric Analyses of Solutions: A spectrophotometer of the Martens polarizing type was employed to analyze the solutions of the pigments. All solutions for spectrophotometric analysis were centrifuged to clarify them just before they were used. Samples showing a tendency toward oxidation by atmospheric oxygen were centrifuged while sealed as described above. They were opened in an atmosphere of nitrogen and the spectrophotometer cell (one cm. in length) was filled and sealed. In this way no contact with oxygen was possible from the time of preparation until the obtaining of the spectrum.

The pH measurements were made with a glass-electrode apparatus. Where particular pH's of solutions were desired 1-to-10 dilutions of Clark-Lubs' (Clark, 1928) buffers were employed as solvents. The particular type of buffer employed for each solution is noted along with the data for that solution.

Spectrum of Nitric Oxide Hemoglobin: In the data obtained for the spectrum of nitric oxide hemoglobin at various pH's (Table 2) the extinction coefficients (ϵ) and concentrations are expressed according to the system suggested by Drabkin and Austin (1935). No significant variation of the spectrum of nitric oxide hemoglobin was obtained over the pH range 5.1 to 8.

Stability of Nitric Oxide Hemoglobin in Absence of Oxygen: Just as there is an equilibrium relationship between oxygen or carbon monoxide and hemoglobin, there undoubtedly is a similar relationship between nitric oxide and hemoglobin. Correspondingly, as the union between carbon monoxide and hemoglobin is stronger than between oxygen and hemoglobin, so is the union between nitric oxide and hemoglobin still stronger, Hartridge (1912). The fact that under

the conditions of preparation of nitric oxide hemoglobin described above it is impossible to pump off nitric oxide from the compound, also argues for a firm bond of combination.

TABLE 2

Spectrum of Nitric Oxide Hemoglobin

Wave length	Buffer								Average
	Phthalate		Phosphate		Phosphate		Borate		
	pH 5.1	Conc.[1] .079	pH 5.75	Conc.[1] .086	pH 7.15	Conc.[1] .072	pH 8	Conc.[1] .109	
mμ	ε		ε		ε		ε		ε
500	7.0		6.8		7.2		7.0		7.0
510	7.0		6.4		7.0		6.7		6.8
520	7.8		7.2		7.8		7.2		7.5
530	10.1		9.5		10.1		9.4		9.8
535	11.5		10.6		11.3		11.0		11.1
538	11.8		11.6		11.9		11.8		11.8
540	12.5		12.1		12.2		12.2		12.3
542	12.8		12.3		12.5		12.6		12.6
544	12.9		12.2		12.4		12.8		12.6
550	12.9		12.1		12.1		12.6		12.4
555	12.4		12.1		11.8		12.0		12.1
560	11.9		11.8		11.7		11.9		11.8
565	12.0		11.6		11.9		11.8		11.8
570	12.1		11.8		12.1		11.9		12.0
572	12.3		11.8		12.1		11.9		12.0
574	12.4		12.2		12.4		12.4		12.4
576	12.3		12.0		12.1		12.1		12.1
578	12.1		11.8		11.8		12.0		11.9
580	11.9		11.5		11.7		11.8		11.7
585	10.4		10.6		10.3		10.4		10.4
590	8.0		8.6		7.9		8.2		8.2
595	5.4		5.8		5.1		5.8		5.5
600	3.7		4.0		3.7		2.9		3.6
610	1.5		1.7		2.0		1.8		1.8
620	0.9		1.1		1.4		1.1		1.1
630	0.5		0.7		1.1		0.7		0.8
640	0.4		0.5		1.0		0.6		0.6
650	0.1		0.3		0.8		0.5		0.4
660	0.1		0.2		0.6		0.4		0.3

[1] Millimoles of nitric oxide hemoglobin equal millimoles of iron.

Despite these considerations it remained possible that the decomposition rate was so slow that, in the absence of appreciable nitric oxide pressure, decomposition might become observable over a longer period of time. To test this, three preparations of nitric oxide hemoglobin at pH's 6.1, 7.1, and 7.9 were sealed under vacuum and stored at 10°C.(50°F.) for about 35 days. At the end of this period the

tubes were opened and the spectra run. From spectra obtained (Fig. 2) it can be seen that no change in the compound occurred. Since the solutions were found to be sterile, bacterial action on the pigment was eliminated. Unquestionably nitric oxide hemoglobin is by all ordinary criteria a stable compound under the conditions of the above experiment, the chief of which are absence of oxygen and bacteria.

Reaction of Nitric Oxide Hemoglobin With Oxygen: Despite the strong bond between nitric oxide and hemoglobin, nitric oxide hemoglobin, unlike the loosely bound compound oxyhemoglobin, is very susceptible to oxidation by atmospheric oxygen. Oxidation of nitric

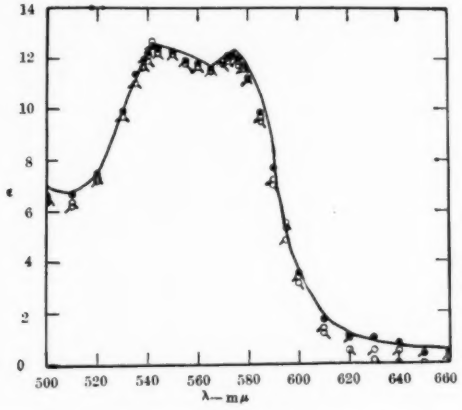

FIG. 2. Curve is smooth curve through average values for NOHb spectrum from data of Table 2. ♀ ε values for preparation of NOHb at pH 6.1 (phosphate buffer) containing 0.077 millimoles NOHb per liter, and stored 34 days under vacuum. ● ε values for similar preparation of NOHb at pH 7.1 (phosphate buffer), containing 0.090 millimoles NOHb per liter and held 37 days. ♀ ε values for preparation of NOHb at pH 7.9 (borate buffer), containing 0.084 millimoles NOHb per liter and stored 36 days.

oxide hemoglobin by oxygen leads to the formation of methemoglobin. The effect of oxidation on the spectrum of a preparation of nitric oxide hemoglobin is shown (Fig. 3). The spectrum observed after oxidation is practically identical with that obtained for methemoglobin at the same pH by Urbain and Greenwood (1940b).

The following experiment was performed in order to determine the effect of oxygen pressure on the oxidation. A preparation of nitric oxide hemoglobin was made at a pH of 6.8 (phosphate buffer) in the apparatus previously described. Small sample tubes having a volume of about 10 ml. were prepared and marks placed on them to indicate approximately five ml. and 10 ml. These tubes were suc-

cessively sealed onto the preparation apparatus at *L* (Fig. 1) according to the method described under the method of preparation of nitric oxide hemoglobin. The tubes were filled as follows: The apparatus was completely evacuated and five ml. of the nitric oxide hemoglobin solution were allowed to flow into the tube from the main chamber, which was then closed off from the rest of the apparatus. A gas (described below) was admitted up to a definite predetermined pressure; the tube was then quickly sealed off and the procedure repeated.

In order to obtain small quantities of oxygen, dilutions of oxygen with inert gases were employed. For this purpose, air (20.9 per cent

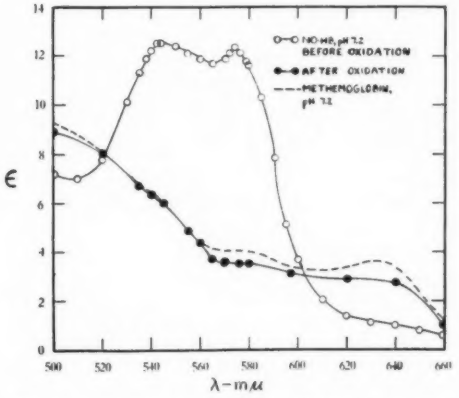

FIG. 3. Conversion of NOHb to MetHb by oxygen.

oxygen) and commercial cylinder nitrogen containing 1.2 per cent oxygen were used. Pure oxygen was used in one series.

The six series of tubes sealed in the above described manner and also the oxygen pressure calculated to exist in each series are listed (Table 3). The preparation of nitric oxide hemoglobin contained .101 millimole nitric oxide hemoglobin per liter. On the basis of five ml. of solution and five ml. of gas above the solution (this ratio of liquid volume to gas volume could be only approximated), the ratio of oxygen equivalents to equivalents of nitric oxide hemoglobin has been estimated and listed (Table 3).

Seven tubes were sealed up for each series. One tube from each series was analyzed spectrophotometrically for oxidation to methemoglobin on the first, second, third, fourth, and seventh days after preparation of the tubes. This was done by obtaining the extinction coefficients at 542, 560, and 575 mμ and calculating, from the

corresponding values of the extinction coefficients for nitric oxide hemoglobin and methemoglobin at the proper pH, the percentage conversion to methemoglobin according to the method described by Austin and Drabkin (1935).

TABLE 3

Data on Oxygen Pressures Existing in Sealed Tubes of NOHb Solution

Series	Average gas pressure	Gas	O_2 pressure	t	Oxygen equivalents	Ratio: Equiv. O / Equiv. NOHb
	mm.		*mm.*			
1	6	Nitrogen	0.072	24	7.7×10^{-9}	0.15
2	24	Nitrogen	0.283	23	30×10^{-9}	0.6
3	93	Nitrogen	1.15	25	12.3×10^{-7}	2.4
4	46	Air	9.7	30	$.102 \times 10^{-5}$	20.1
5	718	Air	151	30	15.9×10^{-5}	313
6	718	Oxygen	718	30	75.8×10^{-5}	1490

Although the method of the experiment limited the quantity of oxygen, it is apparent (Fig. 4) that the rate of oxidation of nitric oxide hemoglobin is influenced by the oxygen pressure. Since nitric oxide itself is capable of oxidation by oxygen, no exact relationship

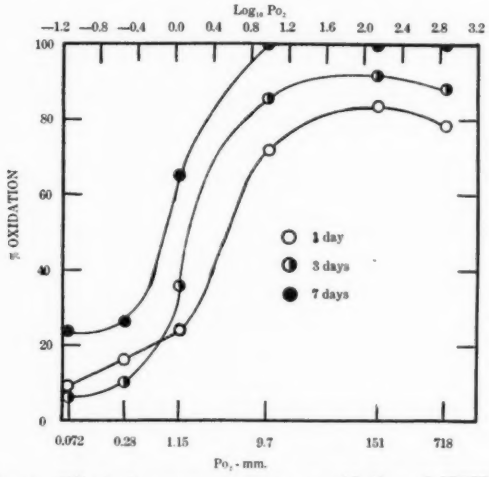

FIG. 4. Effect of oxygen pressure on oxidation of NOHb.

between the quantity of oxygen present and the degree of oxidation of the pigment can be obtained. There exists, however, a rough correlation between the percentage oxidation and the oxygen pressure in each series, and the data (Fig. 4) plainly show that nitric oxide hemoglobin is easily attacked by even small quantities of oxy-

gen, while larger quantities of oxygen rapidly cause complete oxidation. No intermediate optimum pressure is indicated.

Effect of pH and Temperature on Air Oxidation of Nitric Oxide Hemoglobin: Portions of three preparations of nitric oxide hemoglobin were stored as indicated (Table 4); data concerning these preparations are also shown. Access of air was permitted to these solutions, and roughly the same surface of liquid was exposed in each case. At periods indicated (Table 5) samples were withdrawn for the purpose of obtaining the percentage of methemoglobin by spectrophotometric analysis, as described above.

TABLE 4

Data on Solutions for Study of Effect of pH and Temperature on Air Oxidation of NOHb

Preparation	pH	Buffer	Conc.[1]	Temp. of storage
				°C.
1	5.75	Phosphate	.086	0
1	.....	.....	.....	10
1	.....	.....	.....	37
2	6.75	Phosphate	.095	10
3	8.25	Borate	.098	0
3	.....	.....	.....	10
3	.....	.....	.....	37

[1] Millimoles of nitric oxide hemoglobin originally present, or the sum of the nitric oxide hemoglobin and methemoglobin concentrations after oxidation.

A comparison of the data for Preparations 1, 2, and 3 at a temperature of 10°C.(50°F.) shows that oxidation proceeds more rapidly at lower pH's, it being appreciably retarded at a pH of 8.25. Comparison of the three samples from Preparation 1 and also those from Preparation 3 indicates that rise of temperature accelerates the reaction. Where the pH is high and temperature low, the rate of oxidation is comparatively slow, as is seen from Preparation 3 at 0°C. (32°F.) where only 44 per cent oxidation was obtained in 29 days, as compared with 100 per cent in one day for Preparation 1 at 37°C. (98.6°F.).

DISCUSSION

As a meat pigment, nitric oxide hemoglobin has several undesirable features. Chief among these is the ease with which it is attacked by atmospheric oxygen. One saving condition permits the use of nitric oxide hemoglobin as a meat pigment. This is the complete absence of any oxygen tension within meat, as shown by Brooks (1929, 1936). For this reason cured meat may be placed in air with only danger of surface oxidation and discoloration. The mechanism of the oxidation of nitric oxide hemoglobin to methemoglobin appears

to be such that there exists no optimum pressure for the reaction, the rate being merely proportional to the oxygen pressure. This is different from the natural pigment hemoglobin, for whose oxidation Neill and Hastings (1925) and Brooks (1935) found a definite optimum pressure owing to the simultaneous formation of oxyhemoglobin, which is incapable of oxidation. Undoubtedly nitric oxide hemoglobin must first dissociate before oxidation, since hemoglobin in combination with oxygen and carbon monoxide appears incapable of oxidation. The dissociation is probably assisted by the fact that both

TABLE 5

Air Oxidation of Nitric Oxide Hemoglobin

Preparation and temperature	Days					
	1	2	3	6	13	29
	MetHb pct.	MetHb pct.	MetHb pct.	MetHb pct.	MetHb pct.	MetHb pct.
1—0°C.	58	57	58	80	95	88
1—10°C.	53	68	76	88	103	102
1—37°C.	102	100	100	105	104	96
2—10°C.	28	61	69	91	106	100
3—0°C.	3	3	3	4.5	7	44
3—10°C.	5	18	22	52	73	78
3—37°C.	70	....[1]	....	....	....	....

[1] Pigment precipitated. The reason for this is not clearly understood.

products of dissociation, nitric oxide and hemoglobin, are capable of oxidation and consequent removal.

The pH and temperature are factors in the oxidation. The pH of meat is very close to six and is difficult of alteration. The data (Table 5) indicate that it would be necessary to raise the pH considerably above the normal value for meat to inhibit oxidation appreciably. The high temperature coefficient of the oxidation reaction points to a definite need for refrigeration to preserve the color of any surface exposed to air, for instance, sliced bacon. Indeed, brown discolorations observed on sliced cured meats which have been frequently ascribed to bacterial action resulting from improper refrigeration can easily be accounted for in the high temperature coefficient of the oxidation reaction.

SUMMARY

A method of preparing pure nitric oxide hemoglobin by reaction of nitric oxide with hemoglobin is described. The spectrum of nitric oxide hemoglobin over a pH range 5 to 8 is shown to be constant. It is demonstrated that nitric oxide hemoglobin is a stable compound

in the absence of oxygen, but it was found that it oxidized easily by atmospheric oxygen. This oxidation is inhibited by an increase of pH and assisted by rise in temperature.

The relation of the above observations to the color of cured meats is discussed.

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THE HEME PIGMENTS OF CURED MEATS

II. AN APPLICATION OF THE VAN SLYKE-NEILL MANOMETRIC GAS APPARATUS TO THE DETERMINATION OF OXYGEN CAPACITY OF DILUTE HEMOGLOBIN SOLUTIONS[1]

W. M. URBAIN
Research Laboratories, Swift & Company, Chicago, Illinois
AND
D. A. GREENWOOD
Department of Scientific Research, Institute of American Meat Packers, Chicago, Illinois

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In the course of a study of color changes occurring in meat during the curing process, it became necessary to determine the concentration of hemoglobin in dilute solutions. It seemed desirable to do this by two independent methods. The spectrophotometric method described by Drabkin and Austin (1935a) was selected as one method and a modification of the Van Slyke-Neill manometric oxygen-carrying capacity was used as a second method.

The method of Van Slyke and Neill (1924) of determining the hemoglobin content of blood is one of the most reliable methods available, but in the application of it to dilute hemoglobin solutions certain factors must be considered. Sendroy (1931) has extended the use of the Van Slyke-Neill methods to whole blood solutions diluted with isotonic sodium chloride solution. He made a correction for the amount of oxygen physically dissolved in the isotonic salt solution similar to but different from that of the Van Slyke-Neill method for whole blood. This difference was due to differences in the solubility of oxygen in the two solvents, isotonic salt solution and blood. In applying the Van Slyke-Neill method to water solutions of hemoglobin, it would be expected that an entirely new correction for the dissolved oxygen would be necessary.

Recently it has been shown by Sidwell, Munch, Guzman-Barron, and Hogness (1938) that the hemoglobin molecule combines with various anions forming a complex. The oxygen dissociation constants for these complexes are different, and consequently the pres-

[1] This investigation was carried out in the Research Laboratories of Swift & Company, Chicago. The Institute of American Meat Packers collaborated through Mr. D. A. Greenwood, working under a grant to the institute from the Corn Industries Research Foundation.

ence or absence of an electrolyte may affect the determination of hemoglobin by an oxygen-capacity method if complete saturation of the hemoglobin with oxygen is not attainable under the conditions of the determination.

Heidelberger (1922) gives data regarding the effect of time on the oxygen capacity of a solution of oxyhemoglobin which he prepared by a dialysis method. He observed a steady diminution, amounting to roughly one per cent per day in the oxygen capacity of his solutions. Exact data regarding the hydrogen-ion concentration, bacterial population, and storage temperature of solutions were not given.

The object of this paper is twofold: first, to describe a technique of determining the concentration of hemoglobin in dilute solutions using a modification of the Van Slyke-Neill manometric oxygen-capacity technique; second, to report the effect of time and microörganisms on the oxygen capacity of dilute hemoglobin solutions.

EXPERIMENTAL PROCEDURE AND DISCUSSION

Apparatus and Materials: The spectrophotometer used was the same as that described by Urbain and Jensen (1940). An improved Van Slyke manometric blood-gas apparatus was employed in making all oxygen-capacity determinations.

The pH measurements were made with a glass electrode. Numbers of bacteria were determined by making appropriate dilutions of the blood and incubating the organisms at 20°C.(68°F.) for 72 hours, using North Gelatin Agar-Difco as a nutrient medium.

Solubility of Oxygen in Water: Sendroy (1931) indicated that the Van Slyke-Neill manometric technique could be used on whole blood diluted with an isotonic sodium chloride solution, provided that a proper correction was applied for the oxygen physically dissolved in the salt solution at a given temperature and pressure. We, however, desired to use water instead of isotonic salt solution as the solvent. Consequently, it was necessary to determine the amount of oxygen dissolved in water in order to obtain a correction similar to that of Sendroy for isotonic salt solution.

Such a correction could be calculated from the α's [α is the volume of gas, reduced to 0°C.(32°F.) and 760 mm. pressure, dissolved by one volume of solvent when the partial pressure of the gas is 760 mm.] of Winkler (1905) for water as Sendroy (1931) calculated from the data of Geffcken (1904). When this was done, the agreement between the manometric and spectrophotometric methods was unsatisfactory. It was decided, therefore, to determine the solubility of oxygen in distilled water by a modification of the standard Van Slyke-Neill method.

The procedure and reagents used in making the oxygen-capacity determinations were unpublished modifications of the Van Slyke-Neill technique for whole blood employed in the Department of Biochemistry of the University of Chicago, by Dr. Martin E. Hanke.[2] Van Slyke analyses were made either on the whole blood directly

TABLE 1

Solubility of Oxygen From Air in Distilled Water at Various Temperatures and at 740-748 mm. Hg Pressure

Temperature of aeration	Differences in p_1 and p_2 in Van Slyke apparatus, a = 2 ml. S = 7 ml.	Temperature of determination in Van Slyke	Factor for 2-ml. sample in Van Slyke-Neill table	Millimoles O_2 dissolved per liter
°C.	mm.	°C.		
16.0	6.2	17.0	.0554	.343
16.5	6.1	20.0	.0548	.334
20.0	5.6	21.5	.0545	.305
20.0	6.1	22.0	.0544	.332
20.0	5.6	22.4	.0543	.304
22.0	5.8	32.0	.0525	.305
23.0	5.7	33.0	.0524	.299
24.0	5.6	33.0	.0524	.293
25.0	5.2	27.0	.0534	.278
25.0	5.1	26.8	.0534	.272
27.0	5.3	31.0	.0527	.279
27.0	5.0	32.0	.0525	.263
28.0	5.1	31.0	.0527	.269
30.0	4.6	24.8	.0538	.247
30.0	4.6	25.5	.0537	.247
30.0	4.6	26.5	.0535	.246
34.0	4.3	34.0	.0522	.225
34.0	4.4	34.0	.0522	.230
35.0	4.2	34.0	.0522	.219
20.0	24.7	26.0	.0536	.277/.2092[1]
25.5	22.2	25.0	.0538	.249/.2092[1]
29.5	20.6	24.6	.0539	.232/.2092[1]
30.0	20.7	24.5	.0539	.232/.2092[1]

[1] From pure oxygen.

or on dilutions of the whole blood accurately made with distilled water. Purified dilute oxyhemoglobin solutions were also prepared from whole blood by the method described previously by Urbain and Jensen (1940). Spectrophotometric analyses were made on appropriate dilutions with water of the whole blood or of the hemoglobin solutions to yield a satisfactory density reading on the photometer.

[2] We acknowledge our indebtedness to Dr. Hanke for the detailed directions and suggestions.

Distilled water at various laboratory temperatures of 16 to 35°C. (60.8 to 95°F.) was saturated with air by bubbling through it air which had been filtered through cotton. Two-ml. quantities of this water were transferred to the Van Slyke-Neill apparatus and the millimoles of oxygen dissolved in the water were determined. The solubility of pure oxygen in boiled distilled water was determined by a similar procedure. The data obtained are recorded (Table 1 and Fig. 1).

The values for the solubility of oxygen from air in distilled water are somewhat greater than the revised values for the solubility of oxygen in isotonic salt reported by Sendroy, Dillon, and Van Slyke

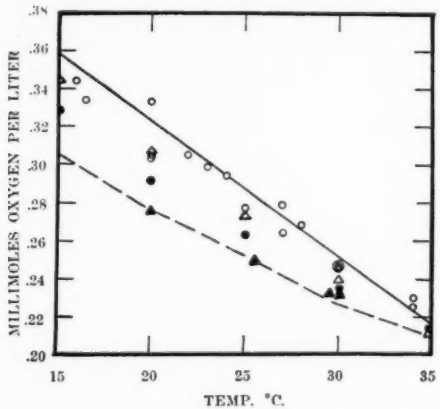

FIG. 1. Solubility of oxygen: O data from Table 1, solubility of oxygen from air in water; ● solubility of oxygen from pure oxygen in .9 per cent NaCl, Sendroy, Dillon, and Van Slyke (1934), calculated to air basis; △ solubility of oxygen from pure oxygen in whole blood, Sendroy, Dillon, and Van Slyke (1934), calculated on air basis; — — — — solubility of oxygen from pure oxygen in water, according to Winkler (1905), calculated on air basis; ▲ solubility from pure oxygen, according to Table 1, calculated on air basis.

(1934). They are also larger than similar values for whole blood given by them. Our values for the solubility of pure oxygen in boiled distilled water are similar to the data obtained by Winkler (1905), also from pure oxygen. Hence, it appears that if air is used to saturate hemoglobin with oxygen, data for the solubility of oxygen physically dissolved from the air should be employed in the correction. Data for the solubility of pure oxygen in water should likewise be used in calculations involving hemoglobin solutions saturated with pure oxygen instead of air.

Modified Van Slyke-Neill Method for Dilute Hemoglobin Solutions: In order to insure equilibrium of the hemoglobin solution

with the oxygen of air and to oxygenate the compound completely, the sample was shaken in the Van Slyke chamber for three minutes in contact with air. When hemoglobin solutions were stored at low temperatures, they were first deoxygenated by the procedure used to prepare oxygen-free reagents and then reoxygenated at the temperature of the Van Slyke chamber. Two-ml. samples of the dilute hemoglobin solution were used in all determinations. In applying the correction for the dissolved oxygen, however, appropriate values from our data (Fig. 1) were employed instead of the usual values of Sendroy, Dillon, and Van Slyke (1934). The proper quantity was subtracted from the total oxygen capacity of the solution in millimoles of oxygen for the temperature at which the solution was equilibrated with air.

Representative data showing a comparison of the values obtained for the hemoglobin concentration by the above manometric method and by the spectrophotometric method are shown (Table 2). The results indicate that hemoglobin solutions could be diluted to at least 1-to-10 and possibly 1-to-20 dilutions with distilled water and still obtain the desired precision (.02 mM. per l.) provided that two-ml. samples were used in making the determinations. These are solutions of sufficient dilution for use in spectrophotometric measurements (employing a one-cm. cell). Some discrepancies occur at higher dilutions, probably owing to limitations of the Van Slyke-Neill apparatus itself.

An additional correction for changes in barometric pressure should be made if the pressure departs significantly from the values 740-748 mm. which prevailed at the time the oxygen-solubility correction was determined (Table 1). Assuming the change in solubility of oxygen in water with change in barometric pressure is the same as that in whole blood, a change of 20 mm. in barometric pressure will cause a change of approximately three per cent in the correction factor, according to the data of Sendroy, Dillon, and Van Slyke (1934). This would amount to less than one per cent error in the determination of the concentration of a solution containing one millimole of hemoglobin per liter.

The data indicate that there is no difficulty in saturating hemoglobin with oxygen under the conditions specified (Table 2). This is in agreement with spectrophotometric data on oxyhemoglobin, especially with those of Drabkin and Austin (1935b) who found that there was no change in the spectrum with wide variations in concentration. Were it impossible to saturate hemoglobin with oxygen at the concentrations listed (Table 2), spectrophotometric data,

which are usually obtained on such dilute solutions, would mean little.

Effect of Time and Microörganisms on Oxygen Capacity: The effect of time and microörganisms on the oxygen capacity of dilute hemoglobin solutions, as determined by the modified Van Slyke-

TABLE 2

Comparison of Modified Van Slyke-Neill Technique and Spectrophotometric Method for Determination of Hemoglobin in Dilute Solutions

Sample	Dilution	Conc. mM. Hb/l.[1]	
		Manometric	Spectrophotometric
Whole Blood			
1a	Whole blood	7.14	7.13
1b	Whole blood	7.14	7.07
2a	1:5	7.15/5	.....
2b	1:5	7.15/5	.....
3a	1:10	7.14/10	.....
3b	1:10	7.14/10	.....
4a	1:20	7.14/20	.....
4b	1:20	7.04/20	.....
5a	1:50	7.80/50	.....
5b	1:50	7.65/50	.....
Purified HbO_2			
6a	Approx. 1:5	1.64	1.64
6b	Approx. 1:5	1.64	1.63
7a	Approx. 1:8	1.21	1.20
7b	Approx. 1:8	1.21	1.20
8a	Approx. 1:8	1.10	1.10
8b	Approx. 1:8	1.09	1.10
9a	Approx. 1:8	1.15	1.15
9b	Approx. 1:8	1.14	1.14
10a	Approx. 1:8	1.04	1.04
10b	Approx. 1:8	1.03	1.04
10c	Approx. 1:16	1.06/2	.....
10d	Approx. 1:16	1.05/2	.....
10e	Approx. 1:32	1.04/4	.....
10f	Approx. 1:32	1.06/4	.....
10g	Approx. 1:64	1.06/8	.....
10h	Approx. 1:64	1.05/8	.....

[1] One millimole of hemoglobin equals one millimole of oxygen capacity.

TABLE 3

Effect of Time and Microörganisms in Oxygen Capacity of Hemoglobin as Determined by Modified Van Slyke-Neill Technique in Dilute Solutions

Days after preparation	Toluene present in HbO_2			Toluene absent in HbO_2		
	Oxygen capacity	pH	Bacteria per ml.	Oxygen capacity	pH	Bacteria per ml.
	mM./l.			*mM./l.*		
SOLUTION A						
0	1.15	7.5	No growth	1.15	7.5	No growth
1	1.15	7.5	No growth	1.15	7.5	Growth
3	1.15	7.5	No growth	1.15	7.5	Growth
4	1.15	7.5	No growth	1.15	7.3	Growth
11	1.15	7.5	No growth	1.15	7.2	Growth
14	1.15	7.5	No growth	0.93	7.2	Growth
18	1.15	7.5	No growth	0.78	7.2	Growth
22	1.15	7.5	No growth	0.67	7.2	Growth
25	1.15	7.5	No growth	0.69	7.2	Growth
29	1.15	7.5	No growth	0.66	7.2	Growth
43	1.15	7.5	No growth	0.57	7.1	Growth
60	1.15	7.5	No growth	0.57	7.1	Growth
SOLUTION B						
0	1.15	7.6	No growth	1.15	7.6	No growth
1	1.15	7.6	No growth	1.15	7.6	Growth
4	1.15	7.6	No growth	1.15	7.6	Growth
8	1.15	7.6	No growth	1.15	7.6	Growth
11	1.15	7.6	No growth	1.15	7.5	Growth
15	1.15	7.6	No growth	1.00	7.5	Growth
29	1.15	7.6	No growth	0.68	7.2	Growth
46	1.15	7.6	No growth	0.57	7.2	Growth
62	1.15	7.6	No growth	0.48	7.1	Growth
SOLUTION C						
0	1.10	7.4	0	1.10	7.4	0
2	1.10	.....	....	1.10	.....	....
4	1.10	7.4	0	1.10	7.4	Less than 10,000
7	1.10	7.4	0	1.10	7.3	....
9	1.10	7.4	0	1.10	7.3	945,000
11	1.10	7.4	0	1.10	7.1	5,880,000
14	1.10	7.4	0	1.03	7.1	17,800,000
16	1.10	7.4	....	1.00	7.1	....
18	1.10	7.4	0	0.92	7.1	25,000,000
21	1.10	7.4	0	0.69	7.1	113,900,000
24	1.10	7.4	....	0.47	7.1	....
28	1.10	7.4	0	0.41	7.1	501,000,000
31	1.10	7.4	0	0.37	7.1	840,000,000

Neill technique, is summarized (Table 3 and Fig. 2). The purified oxyhemoglobin solutions were stored in liter glass bottles at 10°C. (50°F.), and an adequate supply of air was kept over them in order to keep them oxygenated properly. They were shaken daily and fresh air was allowed to enter the bottles. All samples were treated alike, each one being divided into two portions, one of which contained sufficient toluene (five to 10 ml.) to inhibit the growth of microörganisms; the second portion contained no added constituent. The toluene and any denatured protein were removed from the solu-

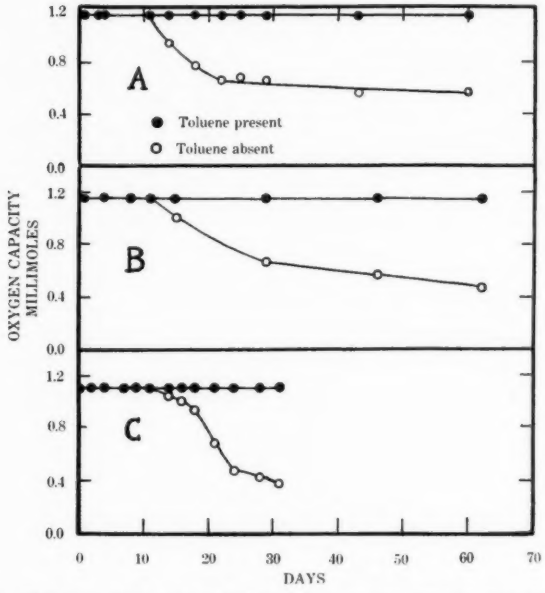

FIG. 2. Effect of time and microörganisms on the oxygen capacity of hemoglobin solutions.

tions before the analyses were made in the Van Slyke-Neill apparatus.

The data indicate that dilute oxyhemoglobin solutions can be stored for 10 or more days in the presence of microörganisms without loss of oxygen capacity at 10°C. Then there was a diminution in the oxygen capacity of the solutions which amounted to about fifty per cent in 60 days or roughly one per cent per day similar to that reported by Heidelberger (1922). The loss in oxygen capacity was likely due to the formation of methemoglobin. It is important to note that the pH of these solutions decreased as numbers of bacteria

increased. The bacteria were a reducing type, and they converted the oxyhemoglobin to hemoglobin, which was oxidized to methemoglobin. The color of the solutions as well as their absorption spectra supported the above explanation.

These data are also in general agreement with the results of Neill (1925a,b,c,d) on the effects of microörganisms on hemoglobin solutions.

It is important to observe that the oxygen capacity of dilute oxyhemoglobin did not diminish for a period of 60 days in solutions which were properly oxygenated and kept free from microörganisms. These data emphasize the importance of keeping hemoglobin solutions free from microörganisms if accurate data regarding this respiratory pigment itself are desired. The course of strictly chemical reactions such as oxidation can be ascertained only if microörganisms are known to be absent. This is particularly true of solutions which are stored several days even at relatively low temperatures. Further, these data point out, as other work has done, that the color of meat in general is affected by the presence of microörganisms acting upon the sensitive heme pigments responsible for the color.

SUMMARY

A new technique has been described whereby the oxygen capacity of hemoglobin diluted with distilled water can be determined by a slight modification of the Van Slyke-Neill method. The modification consists in the dilution of whole blood or purified hemoglobin with distilled water and oxygenation with air. The solubility of oxygen from air in water has been determined by the Van Slyke-Neill technique. These values are used in the correction for the oxygen physically dissolved in the hemoglobin solutions.

Complete oxygenation of hemoglobin up to at least 1-to-10 or 1-to-20 dilutions of whole blood (.70 or .35 millimole of hemoglobin per liter) has been obtained.

Microörganisms and time play an important rôle in causing a loss in the oxygen-carrying capacity of dilute solutions or in the formation of methemoglobin. On the other hand, dilute oxyhemoglobin solutions can be kept for at least two months without a significant loss of oxygen-carrying capacity, provided that they are kept properly oxygenated at low temperatures and microörganisms and products produced by them are absent. Hemoglobin solutions should be kept free from microörganisms if accurate data regarding the pigment itself are desired.

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THE HEME PIGMENTS OF CURED MEATS

III. VARIATION OF ABSORPTION SPECTRUM OF METHEMOGLOBIN IN pH RANGE ONE TO EIGHT[1]

W. M. URBAIN
Research Laboratories of Swift & Company, Chicago, Illinois
AND
D. A. GREENWOOD
Department of Scientific Research, Institute of American Meat Packers, Chicago, Illinois

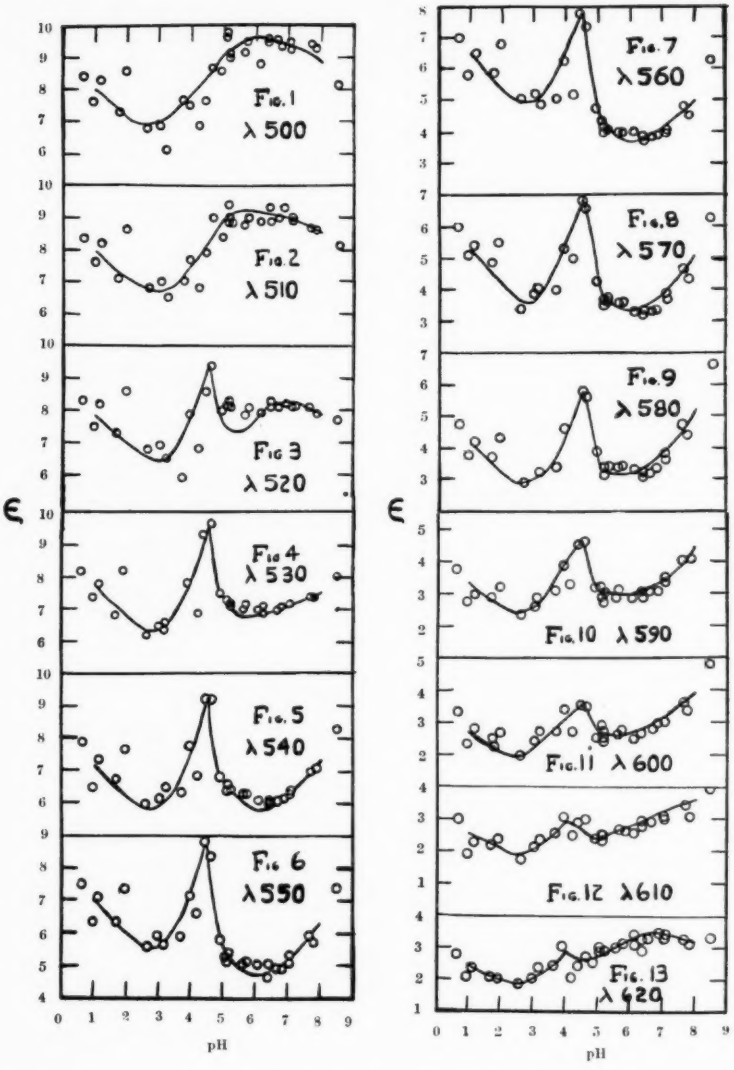
(Received for publication, March 16, 1940)

Methemoglobin is an oxidation product of hemoglobin. Indirectly, it is an oxidation product of several derivatives of hemoglobin among which are oxyhemoglobin and nitric oxide hemoglobin. In a study of the blood pigments of cured meats it was necessary to follow the oxidation of these compounds to methemoglobin; to do this spectrophotometrically, the spectrum of methemoglobin was required. The spectrum of this compound has been obtained by a number of investigators, notably Hari (1920), Haurowitz (1924), and Austin and Drabkin (1935), and it has been shown that methemoglobin has a spectrum which changes with the pH of the solution. Austin and Drabkin (1935) obtained data between pH's 6 and 9.9 and, as previous investigators also proved, showed that methemoglobin in this region acts as an indicator for hydrogen ions; the spectrum obtained at a particular pH is the resultant of an equilibrium mixture of two forms of the pigment.

Drabkin and Austin found that below a pH of 6 their solutions became cloudy and attributed this cloudiness to a proximity to the isoelectric point. Holden (1936) pointed out that they used potassium ferricyanide in the preparation of methemoglobin and ascribed the turbidity below pH 6 to reaction of the pigment with hydroferricyanic acid. Hill and Holden (1927) had previously devised a method of preparation employing ferric tartrate to oxidize hemoglobin, later removing the iron salts. Methemoglobin so prepared did not yield turbid solutions as reported by Drabkin and Austin, confirming Holden's contention.

Holden (1936) investigated the reversibility of the acid denaturation of methemoglobin employing a spectrophotometric method.

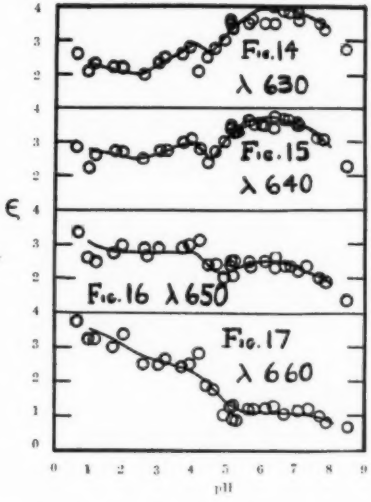
[1] This investigation was carried out in the Research Laboratories of Swift & Company, Chicago. The Institute of American Meat Packers collaborated through Mr. D. A. Greenwood, working under a grant to the institute from the Corn Industries Research Foundation.



His measurements were made in the ultraviolet portion of the spectrum, and his data cover a limited pH range. The purpose of this paper is to report an investigation of the visible absorption spectrum (500 to 660 mμ) of methemoglobin and acid derivatives in the pH range 1 to 8.

EXPERIMENTAL PROCEDURE

Preparation of Methemoglobin: Methemoglobin was prepared from hemoglobin obtained by a method previously described by Urbain and Jensen (1940). To accomplish the oxidation two methods were employed: (a) that of Austin and Drabkin (1935) using potassium ferricyanide and (b) air oxidation for 48 hours at pH 5 and at 37°C.(98.6°F.), according to the method of Coryell, Stitt, and Pauling (1937). The potassium ferricyanide method was employed only on solutions whose pH was greater than 6. The air-oxidation method was employed for all solutions with a pH of 6 or lower and for some above 6. The pH of 5 in the latter method was obtained by



means of a 1-to-10 dilution of a Clark-Lubs' potassium acid-phthalate buffer, Clark (1928); other pH's were obtained from this solution by the addition of measured quantities of either hydrochloric acid (.1 or .5 normal) or sodium hydroxide (.1 normal). Desired pH's of solutions of those preparations made with potassium ferricyanide were obtained with 1-to-10 dilutions of Clark-Lubs' buffers of the proper type for the particular pH. All pH's were measured with a glass electrode.

The air-oxidation method of obtaining methemoglobin is complicated by the necessity of preventing bacterial contamination of the solution prior to and during the "incubation period." The oxidation does not require bacteria but seems to be purely chemical. The presence of bacteria leads to heavy precipitation of pigment difficult

TABLE 1

Rounded Values for Absorption Spectrum of Methemoglobin

pH	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0
mμ	ε	ε	ε	ε	ε	ε	ε	ε	ε	ε	ε	ε	ε	ε	ε
500	8.0	7.6	7.1	6.9	7.0	7.4	7.8	8.4	9.1	9.4	9.6	9.5	9.4	9.2	8.8
510	7.9	7.5	7.2	6.8	6.7	7.0	7.6	8.4	9.0	9.2	9.2	9.1	9.0	8.8	8.5
520	7.8	7.3	6.9	6.6	6.4	6.9	7.9	9.4	7.5	7.3	7.8	8.2	8.2	8.1	7.9
530	7.8	7.2	6.8	6.4	6.4	6.8	7.9	9.7	7.3	6.8	6.8	6.9	7.1	7.3	7.5
540	7.2	6.7	6.3	5.9	6.1	6.5	7.7	9.4	6.7	6.1	5.8	5.9	6.3	6.8	7.4
550	7.0	6.5	6.0	5.6	5.6	6.2	7.2	8.8	5.5	4.9	4.7	4.9	5.2	5.6	6.3
560	6.5	5.9	5.4	5.0	5.0	5.5	6.6	7.8	4.6	3.9	3.7	3.8	4.1	4.5	5.0
570	5.3	4.7	4.2	3.7	3.6	4.3	5.4	6.8	3.9	3.5	3.4	3.5	3.8	4.4	5.1
580	4.2	3.7	3.2	2.9	3.0	3.3	4.4	5.8	3.4	3.2	3.2	3.4	3.8	4.3	5.3
590	3.4	2.9	2.6	2.4	2.6	3.0	4.0	4.6	3.2	3.0	3.0	3.1	3.4	4.0	4.5
600	2.7	2.4	2.2	2.0	2.3	2.7	3.3	3.6	2.7	2.6	2.7	2.9	3.1	3.5	3.9
610	2.5	2.1	1.9	1.8	2.1	2.5	3.1	3.0	2.4	2.6	2.8	3.0	3.2	3.4	3.6
620	2.5	2.2	2.1	1.9	2.1	2.4	2.9	2.7	2.8	3.0	3.3	3.5	3.5	3.4	3.3
630	2.4	2.2	2.1	2.0	2.3	2.6	2.9	2.6	3.2	3.7	3.9	3.9	3.9	3.6	3.4
640	2.8	2.7	2.6	2.6	2.7	2.9	3.0	2.5	3.2	3.6	3.7	3.7	3.6	3.3	2.9
650	3.1	2.9	2.8	2.8	2.8	2.8	2.8	2.3	2.2	2.4	2.5	2.5	2.3	2.1	1.9
660	3.5	3.3	3.0	2.8	2.6	2.5	2.3	1.9	1.3	1.1	1.1	1.1	1.1	1.0	0.9

to remove. The resulting clear solution, in at least one case where bacteria were present, failed to give a spectrum typical of methemoglobin. The presence of a preservative like toluene seems to cause other difficulties. Good preparations were obtained only where technique had prevented bacterial contamination.

The spectrophotometric measurements were made with the same polarizing instrument previously mentioned, Urbain and Jensen (1940). With a few exceptions a one-cm. cell was employed.

Results: The experimental results are shown in the 17 graphs of the extinction coefficient, as defined by Drabkin and Austin (1935), plotted against pH at constant wavelength (Figs. 1-17). Smooth curves were drawn through these points and the rounded values so obtained are listed (Table 1).

DISCUSSION

At no time was a precipitate obtained below pH 6 in those preparations obtained by air oxidation of hemoglobin confirming Holden's (1936) observation regarding the action of potassium ferricyanide in causing the precipitation. In the region where both methods of preparation were applicable, no significant difference in absorption was observed.

These variations of the extinction coefficient at given wavelengths with pH are interesting. An analysis of the ϵ-pH curves indicates that a reversal of the slope occurs for some or all the curves at approximately the pH's 6, 4.5, and 2.75. Two interpretations of the nature of the cause for these reversals are possible.

(1) If we assume two components exist in the pH range 6 to ca. 9, we may postulate additional ones to explain the data for the region below pH 6. Thus in the region 6 to ca. 9, both alkaline and acid methemoglobin exist. The point pH 6 represents the value for complete conversion of alkaline methemoglobin to acid methemoglobin. The rise in absorption between 6 and 4.5 is possibly due to a third compound, with its maximum concentration at pH 4.5 (point of disappearance for acid methemoglobin in the acid range). The fall in absorption after 4.5 requires a fourth compound, which may be acid hematin. Further change in absorption below 2.75 may be due to (a) a fifth compound or (b) a colloidal effect, such as aggregation (the latter effect may enter into the other changes in absorption). Thus we may set up the following chain according to the above interpretation:

A		B		C		D		E
100% pH 9.0		0% ca. pH 9.0		0% pH 6.0		0% pH 4.5		0% 2.75
0% pH 6.0		100% pH 6.0		100% pH 4.5		100% pH 2.75		100% ?
		0% ca. pH 4.5		0% pH 2.75		0% ?		

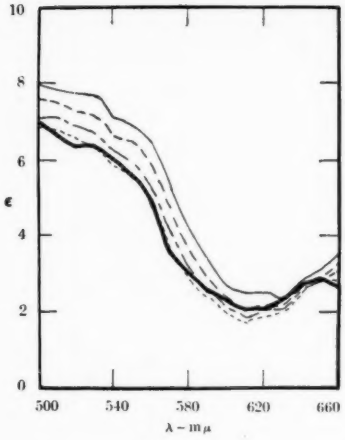


FIG. 18.

——— pH 1.0;
— — — — pH 1.5;
——— – ——— – pH 2.0;
– – – – – pH 2.5;
——— pH 3.0.

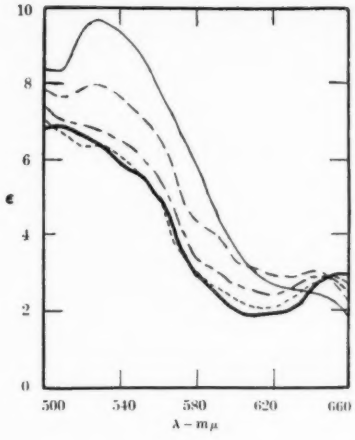


FIG. 19.

——— pH 4.5;
— — — — pH 4.0;
——— – ——— – pH 3.5;
– – – – – pH 3.0;
——— pH 2.5.

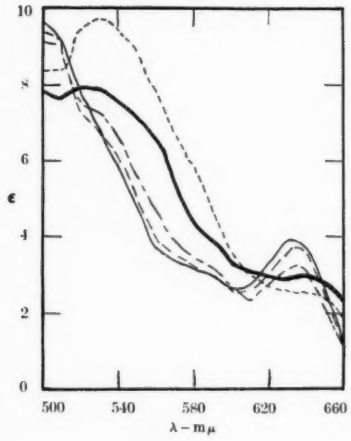


FIG. 20.

——— pH 6.0;
— — — — pH 5.5;
——— – ——— – pH 5.0;
– – – – – – – pH 4.5;
——— pH 4.0.

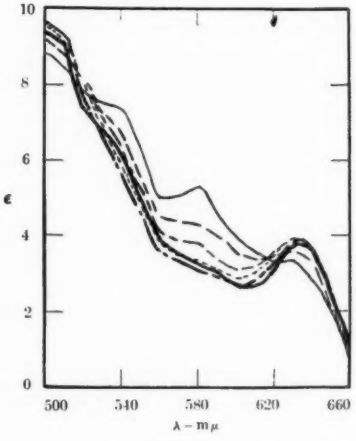


FIG. 21.

——— pH 8.0;
— — — — pH 7.5;
——— – ——— – pH 7.0;
– – – – – – – pH 6.5;
——— – ——— pH 6.0;
——— pH 5.5.

The above interpretation is similar to that of Holden (1936). He states that his data indicate the existence of alkaline methemoglobin (Compound A above) down to pH 6.3. Evidence of denaturation, according to his data, appears at pH 3.9 (corresponding to our value of 4.5) and complete conversion to acid hematin is indicated at pH 3 (our value 2.75). Hence his data and interpretations are similar to those presented above. The present data, however, indicate other intermediate derivatives.

(2) A second interpretation of the change in absorption with pH may be based upon the assumption that the absorption of methemoglobin is altered by changes in the pH without changing the chemical nature of the compound or compounds involved, i.e., the variations observed are not ascribed to the existence of a series of compounds. Such an interpretation, while definitely representing a possibility, lacks support from chemical evidence in at least several instances. It is quite conceivable, however, that some of the inflections of the ϵ-pH curves are due to such an effect, notably the change below pH 2.75, as mentioned above.

SUMMARY

The absorption spectrum of methemoglobin has been obtained in the pH range 1 to 8 for the visible portion of the spectrum.

The variations of the absorption with pH indicate the existence of several absorbing components, one derived from the other as the acidity of the solvent is changed. This is in general agreement with the observations of Holden (1936) obtained in the ultra-violet region of the spectrum.

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THE HEME PIGMENTS OF CURED MEATS

IV. ROLE OF SUGARS IN COLOR OF CURED MEATS[1]

D. A. GREENWOOD AND W. LEE LEWIS
Department of Scientific Research, Institute of American Meat Packers, Chicago, Illinois

AND

W. M. URBAIN AND L. B. JENSEN
Research Laboratories, Swift & Company, Chicago, Illinois

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Traditionally, meat-curing formulas have included certain ingredients. Such curing agents as nitrates and nitrites have been known to be direct factors in good color development. While it has generally been believed, until recently, that materials such as sugar were added solely to improve the flavor of the finished product, recently Lewis (1936, 1937) has shown that the presence or absence of sugar (particularly reducing sugar) in the curing mixture has a direct bearing on the quality of the color obtained at the end of a cure. It was thought that the method of study described previously by Urbain and Jensen (1940) in which the reactions of the isolated meat pigments with various curing agents could be studied, might yield some information as to the manner in which sugars are able to react with the blood pigments and consequently affect the color of the cured meats. The purpose of this paper is to report the results of this study of the role of sugars in color development in cured meats.

EXPERIMENTAL PROCEDURE

The pigments selected for study of these experiments were hog oxyhemoglobin and nitric oxide hemoglobin. They were prepared by the methods described by Urbain and Jensen (1940).

The sugars used in this study were the highest grade manufactured by the Difco Company, except d-tetramethylglucose[2] and crystalline dextrose.[3]

Liter quantities or less of dilute oxyhemoglobin solution were prepared by diluting a stock solution, described previously by Ur-

[1] This work was supported (in part) by a grant to the Institute of American Meat Packers from the Corn Industries Research Foundation.

[2] The d-tetramethylglucose was supplied through the kindness of Dr. M. L. Wolfrom of Ohio State University.

[3] Pure crystalline dextrose was furnished through the courtesy of the Corn Products Refining Company, Chicago.

bain and Jensen (1940), with the desired curing ingredients and distilled water. The pigments were stored in two-liter Erlenmeyer flasks or test tubes stoppered with corks and kept at about 5°C. (41°F.). The following data were obtained on the contents of each flask when the items determined were present in amounts which could be detected quantitatively: millimoles sugar per liter, bacteria per milliliter, pH, concentration of pigment, and colors of solutions with the unaided eye and spectrophotometer. Sugar determinations were made by a modification of the Shaffer-Hartman-Somogyi method for the determination of reducing sugars in blood described by Koch (1937). The modification consists essentially in removing nitrites from the solution by heating the solution in a water bath with dilute sulfuric acid and urea after the protein and other reducing agents have been removed by precipitation with zinc hydroxide. The acid is then neutralized with dilute sodium hydroxide. Sucrose-containing solutions were analyzed for reducing sugar before acid hydrolysis in order that the extent of inversion might be determined during an experiment.

Numbers of bacteria were determined by making appropriate dilutions of the solutions and incubating the organisms at 20°C. (68°F.) for 72 hours, using North Gelatin Agar-Difco as the nutrient medium.

The pH measurements were made with a glass electrode.

Concentration of pigment was determined by the method previously described by Urbain and Jensen (1940).

Color observations were made with the unaided eye and a Martens polarizing spectrophotometer.

Analyses were ordinarily made at frequent intervals over an experimental period comparable to actual meat-curing practice.

When a dilute oxyhemoglobin solution is allowed to stand in a closed container in the presence of certain reducing types of microörganisms, the color of the solution gradually changes from red to purple, indicating that oxyhemoglobin is converted to hemoglobin. This color change generally requires several days at a temperature of 5°C. The microörganisms probably utilize the oxygen in their metabolic processes. It should be noted, however, that oxyhemoglobin solutions do not change color from red to purple when microbial action is inhibited by a substance like toluene.

The sugars—sucrose, maltose, lactose, dextrose, levulose, mannose, galactose, xylose, and d-tetramethylglucose—were added to dilute (Hb .1 mM./l.) oxyhemoglobin solutions in the presence of microörganisms. The solutions were buffered with potassium acid phosphate and adjusted to pH 7 with sodium hydroxide. The color

of the oxyhemoglobin solutions changed from red to purple more quickly in the presence of the above sugars, with the exception of d-tetramethylglucose, than similarly treated oxyhemoglobin solutions containing no sugar. There was a variation in the time (days) required for the different sugars to effect the color change of oxyhemoglobin from red to purple. Tubes containing dextrose, levulose, mannose, galactose, and oxyhemoglobin changed color at about the same time, while sucrose, maltose, lactose, and xylose required a slightly longer period of time to produce the same color change. Tubes containing dilute oxyhemoglobin and d-tetramethylglucose did not change from red to purple until the control tubes containing no sugar had changed color. D-tetramethylglucose, Kendall and Gross (1930), is not utilized by microörganisms, at least by those ordinarily encountered; and the fact that tubes containing this methylated sugar did not change color until the control tubes containing no sugar had changed color, led to the assumption that the shorter time required for the color change of oxyhemoglobin from red to purple was associated with the utilization of the ordinary sugars by microörganisms. Hemoglobin solutions ordinarily become contaminated with adventitious microörganisms unless aseptic technique is followed.

This premise was tested in two different ways. First, toluene was added to another series of test tubes containing dilute oxyhemoglobin and the various sugars described previously. The color of these solutions did not change from red to purple, indicating that the fermentable sugars *per se* did not cause the oxyhemoglobin to change from red to purple. The second method was to add pure fermentable sugars to sterile oxyhemoglobin solutions. Sterile oxyhemoglobin solutions containing dextrose and sucrose were prepared by filtering the solutions through Pasteur-Chamberland filters into sterile flasks. The sterility of the solutions was determined by plating out samples of the pigment solution on North Gelatin Agar-Difco and incubating the samples for 72 hours at 20°C. Sterile oxyhemoglobin solutions containing dextrose and sucrose were kept for 60 days at 5°C. without any significant color change in the pigment. These experiments indicate that the above fermentable sugars cannot affect the color of oxyhemoglobin solutions in the absence of microörganisms or certain metabolic products produced by them.

Similar experiments were performed on purified nitric oxide hemoglobin in the presence and absence of microörganisms and the results confirmed the data obtained with oxyhemoglobin solutions.

The effect of concentration of the fermentable sugar on the rate of color change of oxyhemoglobin from red to purple was investi-

TABLE 1

Effect of Various Concentrations of Dextrose and Sucrose on Dilute Hemoglobin Solutions

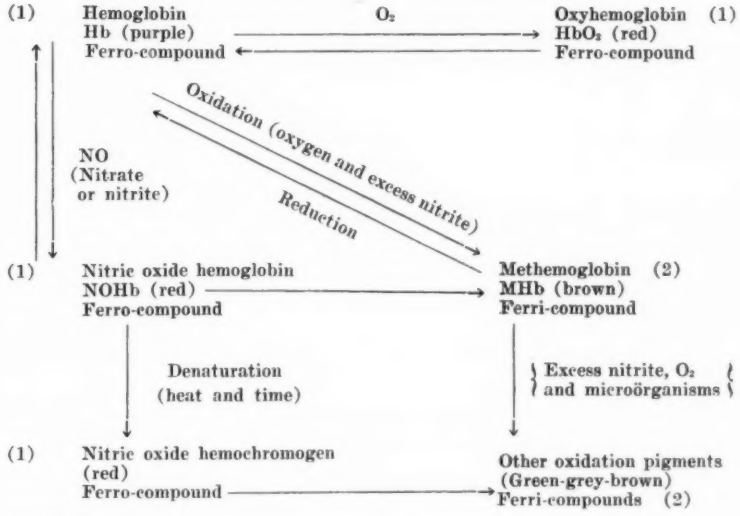
Sample No.	Conc. HbO_2 .100 mM./l.; original pH = 6.9; temp. of refrig. 10°C.(50°F.)			pH at end of 90-day period
	Substances added [1]	Time required for HbO_2 to change from red to purple	Time required for HbO_2 to change from red to brown	
		days	*days*	
127-a	HbO_2	37	Remained purple until end 90-day expt. period.	5.7
127-b	HbO_2 + toluene	No significant color change in 90 days.	No significant change in 90 days.	6.8
127-1	HbO_2 + 10% dextrose	4	15	3.4
127-2	HbO_2 + 10% dextrose + toluene	Same as 127-b	Same as 127-b	6.6
127-3	HbO_2 + 1% dextrose	3	23	3.1
127-4	HbO_2 + 1% dextrose + toluene	Same as 127-b	Same as 127-b	6.8
127-5	HbO_2 + .1% dextrose	3	Same as 127-a	5.0
127-6	HbO_2 + .1% dextrose + toluene	Same as 127-b	Same as 127-b	6.8
127-7	HbO_2 + .01% dextrose	3	Same as 127-a	6.7
127-8	HbO_2 + .01% dextrose + toluene	Same as 127-b	Same as 127-b	6.8
127-9	HbO_2 + .001% dextrose	37	Same as 127-a	6.8
127-10	HbO_2 + .001% dextrose + toluene	Same as 127-b	Same as 127-b	6.8
127-11	HbO_2 + 10% sucrose	4	Same as 127-a	5.0
127-12	HbO_2 + 10% sucrose + toluene	Same as 127-b	Same as 127-b	6.7
127-13	HbO_2 + 1% sucrose	4	Same as 127-a	4.7
127-14	HbO_2 + 1% sucrose + toluene	Same as 127-b	Same as 127-b	6.7
127-15	HbO_2 + .1% sucrose	4	Same as 127-a	5.2
127-16	HbO_2 + .1% sucrose + toluene	Same as 127-b	Same as 127-b	6.8
127-17	HbO_2 + .01% sucrose	4	Same as 127-a	6.8
127-18	HbO_2 + .01% sucrose + toluene	Same as 127-b	Same as 127-b	6.8
127-19	HbO_2 + .001% sucrose	37	Same as 127-a	6.5
127-20	HbO_2 + .001% sucrose + toluene	Same as 127-b	Same as 127-b	6.8

[1] One ml. suspension of a pure bacterial culture was added to each tube and the number of organisms per ml. of solution was about 9,000,000. The organisms were isolated from a commercial pork butt pickle.

gated. Since sucrose and dextrose products are used chiefly in commercial curing practice, pure crystalline sucrose and dextrose were employed in the experiments on the effect of concentration of sugar on the pigment.

The data reveal that oxyhemoglobin solutions containing dextrose changed color from red to purple one day before similarly prepared sucrose solutions (Table 1). It is also important to note that dilute oxyhemoglobin solutions containing one and 10 per cent dextrose

COLOR SCHEME IN MEAT CURING



(1) Desirable pigments.
(2) Undesirable pigments.

changed color from red to purple before solutions containing smaller quantities of dextrose or any concentration of sucrose. The tubes containing one and 10 per cent dextrose changed from purple to brown later, indicating an oxidation of the ferro-hemoglobin to the ferri-hemoglobin derivative, namely, methemoglobin. This oxidative process did not occur to any appreciable extent in tubes containing other concentrations of either sugar. The pH's of the oxyhemoglobin solutions containing one and 10 per cent dextrose were significantly lower than those of other tubes containing smaller amounts of dextrose or the same concentration of sucrose. The color change from purple to

brown in tubes containing one and 10 per cent dextrose can be explained on the basis of the lowered pH of these solutions. The ease of oxidation of ferro-hemoglobin derivatives increases as the pH decreases. Unless sufficiently strong reducing conditions are established simultaneously with the lowering of the pH so that the effect of the pH is offset, the resultant effect of a lowering of pH is one of increased oxidation.

The color changes occurring in this experiment were also followed with the unaided eye and spectrophotometer. Again the results revealed that the color of the oxyhemoglobin solutions containing dex-

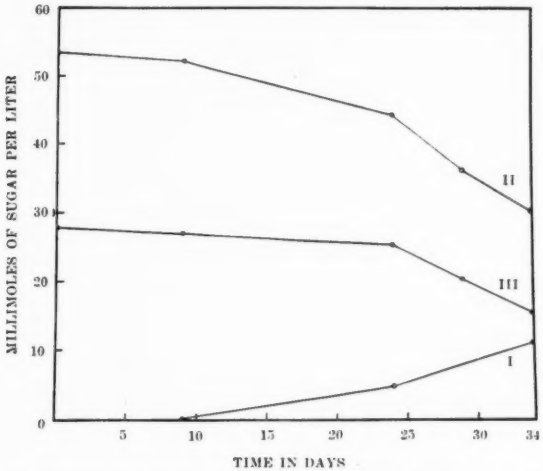


FIG. 1. Utilization and inversion of sugars in dilute oxyhemoglobin solutions.
I. Dilute HbO_2 + .5 per cent peptone + 10 per cent NaCl + sucrose. (Indicating inversion of sucrose *in situ* before acid hydrolysis.)
II. Dilute HbO_2 + .5 per cent peptone + 10 per cent NaCl + dextrose.
III. Dilute HbO_2 + .5 per cent peptone + 10 per cent NaCl + sucrose. (Total sugar after acid hydrolysis.)

trose changed from red to purple before similar flasks containing sucrose or no sugar. As the pH of the sugar-containing solutions decreased, the color of the solutions turned brown, indicating the formation of methemoglobin. It is important to recall that while these solutions were planned to approach meat-curing conditions, they differ considerably from them in buffering capacity and other constituents, such as nitrate and nitrite.

Representative data from a detailed experiment involving the effects of sugars on dilute oxyhemoglobin solutions containing .5 per cent peptone and 10 per cent NaCl are given (Figs. 1, 2, and 3). The

flasks were also inoculated with suspensions of microörganisms from an actual curing pickle. Peptone, sodium chloride, and microörganisms were added to make the solutions more comparable to an actual curing pickle.

The results (Fig. 1) indicate that dextrose and sucrose are gradually utilized by the microörganisms. Dextrose was consumed more rapidly during the early part of the experimental period than sucrose. It is also interesting to note that the inversion of sucrose began about 10 days after the beginning of the experiment and

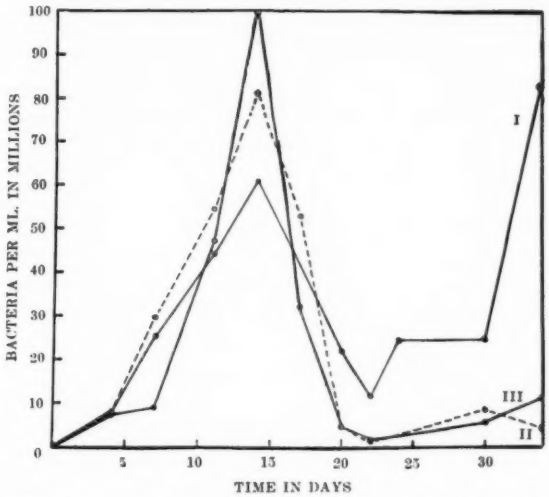


FIG. 2. Effect of sugars on growth of microörganisms in dilute oxyhemoglobin solutions.

I. Dilute HbO_2 + .5 per cent peptone + 10 per cent NaCl.
II. Dilute HbO_2 + .5 per cent peptone + 10 per cent NaCl + dextrose.
III. Dilute HbO_2 + .5 per cent peptone + 10 per cent NaCl + sucrose.

approximately 45 per cent of it was inverted at the end of a 34-day experimental period.

The data (Fig. 2) show the influence of dextrose, sucrose, and no sugar on the growth of microörganisms in dilute oxyhemoglobin solution containing peptone and sodium chloride. The flasks containing glucose stimulated the growth of microörganisms to a greater extent than a similar flask containing sucrose or no sugar during the first 10 days of the experimental period. Flasks containing glucose and sucrose contained a maximum number of microörganisms on the 14th day of the test, with the largest number in the flasks containing sucrose. The rapid growth period was followed by a rapid decline in numbers. The flasks containing no sugar followed

the same population trend as the flasks containing sugar until the 22nd day of the experiment, when the number of microörganisms in the flasks containing no sugar began to increase. The number of microörganisms in flasks without sugar was still increasing at the end of the experiment, while flasks containing sugar remained at a fairly constant low level.

The data (Fig. 3) show that the pH of flasks containing dextrose, oxyhemoglobin, peptone, and sodium chloride decreased more rapidly during the first part of the experimental period than similar flasks

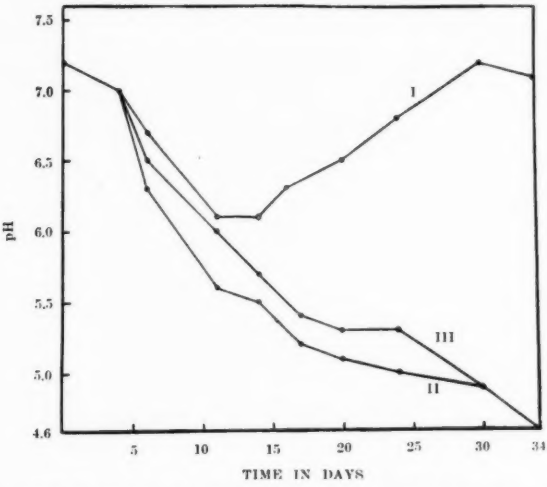


FIG. 3. Effect of sugars on the pH of the dilute hemoglobin solutions.
I. Dilute HbO_2 + .5 per cent peptone + 10 per cent NaCl.
II. Dilute HbO_2 + .5 per cent peptone + 10 per cent NaCl + dextrose.
III. Dilute HbO_2 + .5 per cent peptone + 10 per cent NaCl + sucrose.

containing sucrose or no sugar. The flasks containing dextrose and sucrose reached the same level at the end of the experiment. The results obtained with flasks containing no sugar are of particular interest because the data show that the pH of such flasks decreased until about the 14th day when the pH began to increase significantly. The increase in pH could be correlated with an increase in the number of microörganisms. A putrid odor could be detected in the flask containing no sugar after the 24th day of the experiment, while no putrid odor was observed in flasks containing either sucrose or dextrose. The practical significance of these data in terms of meat curing will be discussed later in this paper.

Neill (1925), Brooks (1934), and Hewitt (1936) have demonstrated that dextrose is capable of establishing reducing conditions in a number of biological systems. Haldane (1901), Jensen (1935), and Brooks (1937) have pointed out the fact that reducing conditions are essential for proper color development in curing meats.

Stone, Wood, and Werkman (1936) have shown that propionic acid bacteria generally activate acetic acid and other low molecular weight fatty acids to donate hydrogen to methylene blue and nitrates. They found that activation of acetic acid, enabling it to donate hydrogen to nitrate, was most pronounced at pH 5 to 6.

The known relationships of the blood pigments involved in the curing of meat are illustrated diagrammatically in the accompanying scheme.

This scheme emphasizes the importance of maintaining reducing conditions during the curing process if desirable cured products are obtained.

Meat is cured in an acid medium in which the pH range of the pickle is from 5.5 to 6.5. This pH range assists in a protein sparing action during the curing process. Lactic acid is formed during the curing process. It is the chief source of the hydrogen ions found in a pickle. Hewitt (1936) states that dehydrogenating enzymes produced by various microörganisms can activate numerous substances to donate hydrogen to a suitable hydrogen acceptor; for example,

$$\underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \underset{\text{Fumaric acid}}{COOHCH:CHCOOH} = \underset{\text{Pyruvic acid}}{CH_3COCOOH} + \underset{\text{Succinic acid.}}{(COOHCH_2)_2}$$

Thus it is possible that substances such as lactic acid can function as an acid and reducing agent in the presence of the proper enzyme systems in the same solution.

The experimental data given in this paper, as well as pertinent observations of other investigators cited, indicate that the addition of sugars, particularly fermentable reducing sugars (dextrose), to solutions containing microörganisms, results in a fairly rapid development of reducing conditions in the system. Several explanations have been offered to account for the phenomenon but the exact mechanism is not known at the present time. This is particularly true of the mechanism by which sugar aids in the establishment of the desired reducing conditions in a complex biochemical system such as meat-curing pickles.

PRACTICAL IMPLICATIONS

The data in the above experiments suggest the following role of sugar in curing meat:

(1) It aids, under certain equilibrium conditions, in improving the color of cured meat by helping to establish reducing conditions when nitric oxide reacts with hemoglobin to form nitric oxide hemoglobin, thus preventing the oxidation of the hemoglobin to methemoglobin.

(2) It tends to prevent oxidation of the ferro-hemoglobin derivatives to ferri-hemoglobin derivatives during storage following the curing process.

(3) It helps to conserve the meat during the curing process by exerting a protein sparing action (Fig. 3).

The results obtained on the isolated pigments have been duplicated under actual curing conditions with meats and led to the same general conclusions listed above.

Sugar and a certain number of desirable reducing microörganisms appear to be essential in curing most meat products. It should be noted, however, that the beneficial effects of sugar and microörganisms can be offset by undesirable colored pigments or cured products if the concentration of sugar and number of microörganisms are not kept within certain limits.

Dextrose can probably be used more readily as a food by more microörganisms than any other sugar used in curing meat. Hence, other factors being the same, solutions containing dextrose and microörganisms will develop the desired pH range and the accompanying reducing conditions more quickly than solutions containing sucrose. The data reveal, furthermore, that oxyhemoglobin solutions containing dextrose at certain concentrations oxidized to the brown methemoglobin more quickly than sucrose-containing solutions. The results indicate that to produce optimum conditions for color fixation, dextrose or a mixture of dextrose and sucrose should be used where a quick cured product is desired. In long cures (30 to 50 days) sucrose is inverted to dextrose and levulose; hence, a mixture of reducing sugars is present at the end of a long cure. Even in the case of long cures, however, the presence of reducing sugars at the very beginning of the cure helps to insure the presence of reduced hemoglobin which reacts with nitrite to form nitric oxide hemoglobin without the accompanying formation of undesirable methemoglobin.

SUMMARY

Dextrose, levulose, mannose, galactose, xylose, sucrose, maltose, and lactose produced significant changes in the color and other characteristics of the blood pigments, oxyhemoglobin and nitric oxide hemoglobin, in the presence of microörganisms. The color changes of the pigments could be correlated with the growth of microörganisms

which utilize the sugars, yielding acid products, and which also aid in establishing reducing conditions. The complex chemical changes observed in flasks containing the blood pigments, fermentable sugar, and microörganisms were in a state of dynamic equilibrium.

Reducing sugars, such as dextrose and levulose, which are readily used by microörganisms, produced color changes in the blood pigments more quickly than sugars which are less readily metabolized by microörganisms. The color changes observed in most of these experiments are typical of color changes occurring in the actual meat-curing process. Large concentrations of a readily available reducing sugar (dextrose) in the presence of large numbers of organisms caused undesirable color changes in the pigments owing to the lowering of the pH of such solutions to a level where ferro-hemoglobin derivatives were oxidized to the ferri-hemoglobin compound, methemoglobin.

Sugars appear to exert no action on the blood pigments in the absence of microörganisms or substances produced by them.

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PROPERTIES OF THE OXIDIZING ENZYMES OF CERTAIN VINIFERA GRAPES[1]

A. A. HUSSEIN AND W. V. CRUESS
Fruit Products Division, University of California, Berkeley, California

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White grapes darken during drying and subsequent storage of the resulting raisins; the freshly expressed, unheated juice becomes brown on standing; and white wines slowly darken during aging. Since it appears that oxidizing enzymes are involved, at least to a considerable extent, in these changes, the investigation reported in this paper was undertaken upon some of the properties of the oxidizing enzymes occurring in certain Vinifera grapes, particularly of the Sultanina (Thompson seedless) variety.

REVIEW OF LITERATURE

Martinand (1895) was one of the first to report that the darkening of the coloring matter in wine is caused by oxidation, catalyzed by a soluble ferment termed "diastase," which in a later paper (1897) he considered to have the properties of a laccase. Since mold growth is often present on French grapes, however, it is possible that he may have confused the oxidizing enzyme of the fungus with that of the grapes. Bertrand (1896) applied the name "oxidase" to oxidizing enzymes in order to distinguish them from other enzymes. Raper (1928) pointed out that guaiacum cannot be used safely as a criterion for the presence of any particular oxidizing enzyme, as no less than five plant-oxidizing systems react with it. Szent-Gyorgy (1925) suggested that the action of direct and indirect oxidases on guaiacum could be explained by the formation of peroxides by autoxidizable substances. Onslow (1919) held similar views and preceded Szent-Gyorgy in that respect. Richter (1934), Elliot (1932), Samisch (1934), and Dixon (1934) contributed further knowledge concerning these enzymes. Cruess and Fong (1926, 1929a, b) and Overholser and Cruess (1923) reported on the nature of the oxidizing enzymes of several fruits and on the action of SO_2 on fruit oxidase. Kertesz (1933, 1934) made interesting observations on the browning of peach tissue. Cruess, Quin, and Mrak (1932) made observations on the browning of canning peaches and upon

[1]The research reported in this paper was aided in part by nontechnical assistance provided under WPA Project No. 11589-B5 and 10481-B5, Area 8, Northern California (Fruit Products Division).

the death temperature of the peach oxidase. Balls and Hale (1934a, b, 1935) reported upon the nature and reactions of peroxidase and upon the peroxidase of apple tissue. Keilin and Mann (1937) have recently obtained highly purified peroxidase preparations in which they find that their enzymes were hematin complexes. Bach and Chodat (1908) considered an oxidase to consist of a peroxidase and an oxygenase, the latter being the portion replaceable by H_2O_2. Onslow (1919) considered oxidase to be a direct oxidizing system capable of bluing gum guaiacum without addition of H_2O_2. She stated also that in plant tissues containing such enzyme systems there is always present an aromatic compound with a catechol grouping.

Wilstätter and Webber (1926) considered peroxidase as the enzyme that causes oxidation of certain phenolic substances, for example, pyrogallol in the presence of H_2O_2 to purporogallin. It has been rather generally considered that a plant enzyme that will catalyze this particular oxidation is a peroxidase. Dixon (1934) holds that catalysis of oxidation of guaiacol in the presence of H_2O_2 is also a criterion of peroxidase activity.

Balls and Hale (1935), previously mentioned, reported that the oxidase that catalyzes the browning of apple tissue is a peroxidase. Balls' method of estimating peroxidase activity quantitatively involves catalysis of the reaction of H_2O_2 and pyrogallol by the enzyme preparation under specified conditions and determination of the unused H_2O_2 by iodine titration at the end of the reaction period. Overholser and Cruess (1923) reported that the oxidizing system of the apple included a peroxidase. Raper (1928), Szent-Gyorgy (1925), and many others have contributed to the literature on peroxidase and fruit-oxidizing systems but space does not permit their review.

EXPERIMENTAL PROCEDURE

Location of Enzyme in the Grape: Keeble and Armstrong (1912) found in *Primula sinensis* that peroxidase concentration was highest in the vascular bundles and epidermis; Christ and Batjer (1931) found oxidase concentration higher in the vascular bundles than in the surrounding tissue; and Samisch (1935b) found oxidase activity in the apricot fruit highest in the stomatal guard cells of the epidermis and in cells surrounding the fibrovascular bundles.

In grapes we found by immersing the cut grape tissue in slightly oxidized benzidine solution the greatest oxidase activity to be in the epidermal cells, although activity was also strong in the vascular bundles. The pericarp showed much less activity.

Preparation of Enzyme Extract: In order to secure an enzyme preparation sufficiently clear to permit observation in a colorimeter,

the following procedure was used in preparing our peroxidase extract:

Grapes or fresh-grape pomace (press cake) were frozen at —17.8°C.(0°F.), ground with white sand in a mortar with acetone, filtered, and the grinding and filtration were repeated at least twice. The solid residue was then extracted with citrate buffer solution of pH 4.5 equal in volume in cubic centimeters to three times the weight of the original grapes in grams.

Three volumes of 95 per cent ethyl alcohol were added, centrifuged, and the precipitate suspended in citrate buffer. This was repeated three times and the solution filtered. The dilute acetone checked browning, removed autoxidizable catechol compounds, tannins, and most of the water-soluble materials; while at the same time it precipitated the enzyme.

Action on Various Substrates: To small amounts of the clear enzyme extract various substrates were added, in one case without H_2O_2, in the other with a small amount of dilute H_2O_2 solution. Development of the characteristic color of the oxidized substrate was taken as evidence of catalytic action. These tests were repeated with all of the substrates, using the boiled enzyme extract, in order to determine the extent of autoxidation of each substrate. All solutions were buffered to pH 5.5.

Of the 27 substrates tested, only catechol, protocatechuic acid, gum guaiacum, o-phenylene diamine, o-aminophenol, and 2-amino-4-nitrophenol developed coloration without addition of H_2O_2, the color being about as intense as that with the boiled enzyme extract with the last-named substance.

With H_2O_2 the following substrates developed characteristic coloration or precipitate: o-cresol, p-cresol, alpha-naphthol, beta-naphthol, guaiacol, catechol, protocatechuic acid, hydroquinone, pyrogallol, gallic acid, tannic acid, gum guaiacum, o-phenylenediamine, p-phenylnediamine, m-phenylenediamine, benzidine, o-aminophenol, m-aminophenl, p-aminophenol, and 2-amino-4-nitrophenol. The following substrates failed to develop color: m-cresol, phenol (C_6H_5OH), resorcinol, phloroglucinol, tyrosine, orcinol, and tryptophane.

As previously stated, our preparation appears definitely to contain a peroxidase. It differs from horse-radish peroxidase in at least one respect, in that the latter, according to Elliott (1932), oxidizes resorcinol, while our preparation did not do so. It also oxidized guaiacol in the presence of persulfate. It did not cause coloration of tyrosine and, therefore, appears not to contain a tyrosinase. Neither the addition of catechol nor of vitamin C (ascorbic acid) to the extract caused coloration of tyrosine.

Since many phenolic substances developed color in the presence of the enzyme extract and H_2O_2, we may conclude that the preparation contains a phenolase which may or may not be identical with the peroxidase.

Natural Substrate: The natural substrate of Sultanina grapes was concentrated by acetone extraction, precipitation with lead acetate, liberation by dilute H_2SO_4, filtration, and vacuum concentration. It gave the usual color reactions for catecholic substances and became brown when allowed to stand in air with the enzyme preparation. It also gave, after such reaction, positive tests for orthoquinone. The mixture of enzyme extract and natural substrate was capable of

TABLE 1

Effect of Time on Color Production

Time	Log 1/T	$\text{Log}\frac{a}{a-x}$	$K_1 = \ln\frac{a}{a-x} \times \frac{1}{t}$
min.			
10	.048	.022	.0051
20	.089	.040	.0046
30	.140	.065	.0050
40	.179	.086	.0050
50	.226	.112	.0052
60	.226	.134	.0052

reacting with starch iodide indicator; that is, it was a "complete oxidizing system."

Quantitative Measurement of Grape-Peroxidase Action: After comparison of the methods of Balls, Willstätter, and others it was found that the guaiacol colorimetric technique of Bansi and Ucho (1926) gave the most consistent and reproducible results. Although it caused darkening of pyrogallol, our preparation used so little H_2O_2 that it could not be estimated. As modified, the procedure is as follows: one c.c. of enzyme extract and one c.c. of a one-per cent guaiacol solution were added to five c.c. of .2 M acetate buffer of pH 4.5 in a test tube and one c.c. of .1 normal H_2O_2 was added. Distilled water was then added to bring the volume to 20 c.c. The tube was held at 30°C.(86°F.) for one hour. The reaction was stopped by addition of five c.c. of glacial acetic acid, as recommended by Van der Veen (1933) instead of the mineral acids or $HgCl_2$ used by Bansi and Ucho, as the acetic acid did not cause bleaching of the color or clouding of the solution in 45 minutes. An Evelyn photoelectric colorimeter was used for all color comparisons, using a light screen of 5400 Å units. With the enzyme preparation acetate buffer of pH 4.5 was used in most experiments because it is near the opti-

mum pH for enzymic oxidation of guaiacol. The color formed obeyed Beers' law. The transmission, T, of light of 5400Å was transformed to $\log \frac{1}{T}$.

Effect of Time on Color Production: Tubes of the reaction mixture, prepared as previously described, were allowed to incubate at 30°C. and color readings were made at intervals of 10 minutes (Table 1). A straight line is obtained by plotting time against $\log \frac{1}{T}$; also by plotting $\log \frac{a}{a-x}$ against time. The latter relationship indicates that the color formation is in the nature of a first-order reaction. The constancy of K_1 over the period used (60 minutes) also indicates a first-order reaction.

TABLE 2

Effect of Enzyme Concentration on Rate of Oxidation of Guaiacol

Enzyme concentrations per 100 c.c.	$\text{Log} \frac{1}{T}$	K
c.c.		
1.5	.031	2.12
3.0	.044	2.12
5.0	.105	2.10
10.0	.208	2.08
15.0	.288	1.92
20.0	.352	1.76
25.0	.426	1.70

Effect of Enzyme Concentration: It was observed that the rate of oxidation of guaiacol within certain limits varied directly with the enzyme concentration, the value of K remaining constant over much of the range used, where $K = \log \frac{1}{T\,C}$. In this case C is the concentration of the enzyme extract. At fairly high concentrations, however, the increase in rate per unit increase in enzyme concentration became less and the K value dropped progressively (Table 2).

Effect of pH Value: The rate of oxidation as measured by readings made as previously described indicated that the optimum pH range was about pH 4.5 to 5.5. This is in fair agreement with the pH optimum of 5.2 for horse-radish peroxidase plus guaiacol as determined by Bansi and Ucho (1926), and of pH 5 to 5.5 for the apricot enzyme by Cruess and Fong (1929a); however, the optimum

range will vary with the substrate used. The accompanying graph illustrates the relation of amount of color formed per unit time and the pH value.

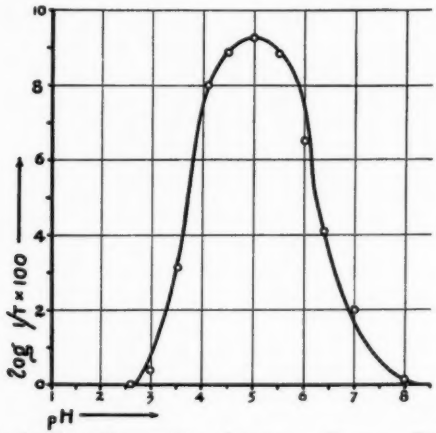

FIG. 1. Effect of pH on the activity of enzyme. (Source: Tokay grapes.)

Effect of H_2O_2 Concentration: It was found that at concentrations much above 5×10^{-4} mols per liter the rate of oxidation decreased materially. Thus, taking the activity at 2.5×10^{-4} mols per liter as 100, the activity at 1×10^{-3} mols per liter was 86; at 2.0×10^{-3} it was 59; at 5×10^{-3} it was 35; and at 7.5×10^{-3} it was 31. The corresponding values of $\log \frac{1}{T}$ were .490, .420, .290, .170, and .155, respectively. A concentration of 1×10^{-3} mols per liter is 34 milligrams of H_2O_2 per liter or 3.4 mgm. per 100 c.c. It is essential that the H_2O_2 concentration be closely controlled.

Effect of KCN: Inactivation of oxidase by KCN has been used as a criterion of the presence of an oxygen-activating rather than hydrogen-activating enzyme. Fifty per cent of the activity of horseradish peroxidase is destroyed by 5×10^{-6} M KCN, Hand (1933) and Dixon and Elliott (1929). The effect of various concentrations of KCN on the activity of our enzyme preparation is shown (Table 3). Evidently our preparation contained almost exclusively the oxygen-activating type of enzyme, that is, peroxidase.

Dehydrogenase Tests: In the presence of ascorbic acid in a Thunberg tube under high vacuum the enzyme preparation failed to cause any bleaching of methylene blue in 24 hours. Neither freshly expressed juice nor fresh grape tissue exhibited any noticeable de-

hydrogenase activity with methylene blue and ascorbic acid or citric acid. These observations would indicate that neither the enzyme preparation nor the fruit contained dehydrogenase.

Action on Ascorbic Acid: The plant-oxidase systems studied by Zilva and Johnson (1937), Szent-Gyorgy (1928), and Srinavasian

TABLE 3

Effect of KCN on Activity of Grape Peroxidase

KCN molality	Log 1/T	Activity
		pct.
0	.370	100.0
2.5×10^{-5}	.190	51.4
5×10^{-5}	.030	8.1
1×10^{-4}	.020	5.4
2×10^{-4}	.015	4.05
3×10^{-4}	.006	1.30
4×10^{-4}	0	0

(1936), oxidized ascorbic acid. Following the technique of Srinavasian we found no oxidation of ascorbic acid by our enzyme extract during 75 minutes' incubation at 37°C.(98.6°F.). However, in the presence of catechol, ascorbic acid was oxidized rapidly. A control was made with buffer solution held at 37°C.; the data appear

TABLE 4

Oxidation of Ascorbic Acid by Enzyme Extract in Presence of Catechol

Time	Ascorbic acid oxidized
min.	*pct.*
2	28
5	38
10	46
15	54
20	72
25	86
30	98
35	100

(Table 4). It is interesting that no coloration from oxidation of catechol to orthoquinone appeared until all the ascorbic acid had disappeared.

Effect of Fluoride Ion: At 4×10^{-4} molal concentration of NaF the enzyme preparation exerted only 47 per cent of its normal activity; at 1.6×10^{-3} M, only 25 per cent; and at 1×10^{-2} M, it was completely inhibited. Samisch (1934) obtained similar results with

apricot oxidase. We were unable to test iodide, bromide, and chloride ion by our technique since in the presence of acid and H_2O_2, liberation of free iodine, chlorine, and bromine occurred.

Effect of Alcohol: Wines contain from about 10 to 22 per cent of alcohol. It was considered of interest to determine to what extent moderate concentrations of alcohol affect the activity of our enzyme preparation. The data indicate (Table 5) that in wines of normal alcohol content, 10 to 21 per cent, the naturally occurring enzyme could exert oxidative action. In other observations we found that new, commercially prepared, white wines in some cases gave a positive test for oxidase with benzidine and darkened slowly on standing

TABLE 5

Effect of Alcohol on Grape-Oxidase Activity

Alcohol by volume	Activity
pct.	*pct.*
0	100.0
5	94.0
10	83.5
15	73.5
20	63.25
26	49.0
30	31.0
35	25.4
40	16.3
50	5.0
60	0.0

in the presence of oxygen. Darkening also proceeded, although more slowly, in the complete absence of the enzyme.

Effect of Sugar Concentration: Data are presented illustrating the effect of added cane sugar on the activity of the enzyme preparation (Table 6). Owing to the fact that added sucrose increased the enzyme activity at concentrations up to 50 per cent the experiment was repeated with a second enzyme preparation, which fact accounts for Series 1 and 2. It is interesting that the maximum stimulating effect of the sucrose occurred between 20 and 30 per cent, the range of sugar content of ripe grapes in California. We have no explanation for this observed effect. Beyond 50 per cent sugar the rate of reaction was retarded.

Effect of SO_2: To portions of the enzyme preparation SO_2 was added to give concentrations ranging from 0 to 5,580 parts per million. The solutions were allowed to stand 10 minutes and then H_2O_2 was added. The activity of the enzyme was then determined. It was

observed that a progressive decrease in enzyme activity occurred with increase in SO_2 concentration. The enzyme preparation previously treated at the maximum concentration of 5,580 parts per million (.558 gram per 100 c.c.), showed an activity of 5.6 compared with 100 for the untreated check. Evidently the SO_2 weakened the enzyme either by reacting with it directly, or by virtue of the effect of increased hydrogen-ion concentration. All lots were buffered to pH 4.5 before addition of guaiacol. See also Fong and Cruess (1929).

Effect of Proteolytic Enzymes: The grape-enzyme preparation on treatment with papain at pH 5, or with trypsin at pH 7 at 37°C.

TABLE 6

Effect of Sucrose Concentration on Activity of Enzyme

Sucrose concentration	Log 1/T		Activity	
	Series 1	Series 2	Series 1	Series 2
pct.				
0	.1020	.096	100.0	100.0
10	.1250	.115	122.0	120.0
20	.1380	.131	135.0	136.0
30	.1280	.122	126.0	129.0
40	.1220	.111	120.0	116.0
50	.1080	.099	106.0	103.0
60	.0814	.076	82.5	78.0
70	.0517	.053	56.0	55.0
80 [1]	.0218	.024	27.5	25.5

[1] Supersaturated.

(98.6°F.) for 15 hours, lost its power to oxidize guaiacol in the presence of H_2O_2. At other pH values of similar level the activity of the grape enzyme was greatly diminished, in comparison with the untreated. These results would indicate that the grape enzyme or its carrier is of protein nature and susceptible to attack by papain and trypsin.

Inactivating Temperature: By placing small volumes of the enzyme preparation in various media in thin-walled test tubes into a large bath of water held at constant temperature, the death temperatures at several pH values and at five-minute holding periods were found to be as follows: in grape juice at pH 4, approximately 85°C. (185°F.); in wine at pH 3.1, approximately 85°C.; and in buffer solution at pH 5.2, 90°C.(194°F.).

Effect of Temperature on Rate of Reaction: The activity of the enzyme preparation was determined at the several temperatures shown (Table 7). The data indicate that for the conditions of this experiment the optimum range was about 33 to 40°C.(91.4 to 104°F.),

TABLE 7

Effect of Temperature on Activity of Enzyme

Temperature	Log 1/T	Activity
°C.		
0	.068	14
5.0	.086	21
10.4	.142	35
30.0	.335	83
33.4	.372	92
36.3	.405	100
40.0	.352	87

while 40°C. was slightly above and 33.4°C. slightly below the optimum.

SUMMARY AND CONCLUSIONS

A method of securing a satisfactory oxidizing-enzyme preparation from white grapes was devised.

The method of estimating the enzyme's activity was by measurement of depth of color formed by action upon guaiacol in the presence of H_2O_2. A straight line was obtained in plotting $\log \frac{1}{T}$ against time when T is the light transmission.

The enzyme concentration was higher in the fibrovascular bundles and epidermis than in the pericarp.

A number of substrates were acted upon positively by the enzyme preparation, among these pyrogallol in the presence of H_2O_2. Tyrosin was not acted upon. The natural substrate of the grapes was purified and when added to the enzyme preparation reacted even in the absence of H_2O_2, agreeing with Onslow's results (1931) with other fruit.

At low to medium concentrations of the enzyme the color produced in unit time varied approximately directly with the enzyme content of the solution.

The optimum pH range under the conditions of our experiments and using guaiacol as the substrate was about pH 4.5 to pH 5.5.

H_2O_2 concentration markedly affected the enzyme activity.

Over 90 per cent of the activity of the enzyme was destroyed by 5×10^{-5} molal KCN, and 100 per cent destruction occurred at 4×10^{-4} molal concentration. These observations and other properties of the enzyme lead us to classify it as a peroxidase.

No reduction of methylene blue was secured with various substrates, using the Thunberg technique. Apparently the preparation contained little to no dehydrogenase.

In the absence of catechol and its derivatives ascorbic acid was not oxidized; but in the presence of catechol the acid was rapidly oxidized, although no color of orthoquinone appeared until all of the ascorbic acid had disappeared.

Fluoride ion strongly inhibited the enzyme.

At 60 per cent by volume of ethyl alcohol the enzyme was completely inhibited as judged by color formation with guaiacol in a limited incubation period; but activity was well retained at 10 to 20 per cent alcohol, the usual range for wines.

Sucrose caused an increase in the enzyme's activity between 10 and 50 per cent sucrose, with greatest activity between 20 and 30 per cent. Activity was decreased at 60 to 80 per cent, but even in saturated sucrose solution was still appreciable.

Contact with SO_2 reduced the enzyme's activity, progressively with increased SO_2 concentration, but even at 5,580 parts per million of SO_2 the enzyme was not completely destroyed.

Papain and trypsin at their optimum pH values, 5 and 7 respectively, destroyed the enzyme.

The optimum temperature range appeared to be in the neighborhood of 33 to 40°C.(91.4 to 104°F.).

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